Analysis of Memory Consolidation and Reconsolidation in the Mouse Hippocampus

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To My Parents

Helena & Hannu von Hertzen

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ABSTRACT

Our brain processes enormous amounts of information subserving the formation and retrieval of memory. A fresh memory is an unstable trace that is stabilised by a process termed consolidation. This depends on de novo transcription. Reactivating a stabilised memory returns it to a labile state, and triggers a second consolidation process, termed reconsolidation, re-stabilising the memory again.

This study used two transcriptional screens to determine the molecular relationship between consolidation and reconsolidation. Three immediate-early genes were identified to be regulated in the hippocampus after contextual fear conditioning in the mouse: Serum and Glucocorticoid regulated Kinase 1 (SGK1), SGK3 and Nerve Growth Factor Inducible gene B (NGFI-B). The up-regulation of SGK1 expression was triggered by the environment, and thus SGK-1 might be important for the formation of a contextual representation. The up-regulation of SGK3 and NGFI-B expression was specific to the context-shock association, suggesting a role for these genes in the consolidation of the context-shock association.

Re-exposure of the mice to the context reiterated the upregulation of SGK1 and SGK3 expression, demonstrating that some transcriptional events after contextual conditioning are recapitulated during reconsolidation, and showing for the first time that an expression change specific to the context-shock association (SGK3) can be subsequently recapitulated during reconsolidation. It was also established that the transcriptional changes induced by retrieval depended on the remoteness of the reactivated memory.

In contrast to SGK1 and SGK3, NGFI-B was not regulated during reconsolidation, and therefore was specific to memory consolidation. This consolidation-specific regulation of NGFI-B was confined to hippocampal area CA1 and required αCaMKII autophosphorylation. This finding suggests a link between synaptic activity and gene regulation in memory formation. Functional studies of NGFI-B showed that NGFI-B was

important for hippocampus-dependent memory formation. The discovery of this consolidation-specific transcript indicates that reconsolidation is only a partial recapitulation of consolidation at the transcriptional level.

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AL	adjacent left quadrant
AM	affymetrix microarray
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isooxazoleproprionic acid
ANOVA	analysis of variance
AR	adjacent right quadrant
BDNF	brain-derived neurotrophic factor
CA	*cornu Ammonisi*
Ca^{2+}	calcium ion
CaM	calmodulin
CaMK	calcium/calmodulin dependent protein kinase
CaMKK	calcium/calmodulin dependent protein kinase kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
C/EBPβ	CCAAT/enhancer-binding protein beta
CR	conditioned response
cRNA	complementary ribonucleic acid
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CS	conditioned stimulus
DD	differential display
DG	dentate gyrus
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded deoxyribonucleic acid

DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Elk-1/SRF	Ets-like transcription factor/serum response factor
EPSP	excitatory postsynaptic potential
EST	expressed sequence taq
FRET	fluorescence resonance energy transfer
IEG	immediate early gene
L&M	learning and memory
LTM	long-term memory
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
Mg^{2+}	magnesium ion
MM	mismatch
mRNA	messenger ribonucleic acid
MWM	Morris water maze
Na^{+}	sodium ion
NF-kB	nuclear factor kB
NGFI-B	nerve growth factor inducible gene B
NMDA	N-methyl-D-aspartate
OP	opposite quadrant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PM	perfect match
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription

SDS	sodium dodecyl sulphate
SGK	serum and glucocorticoid regulated kinase
STM	short-term memory
ssDNA	single stranded deoxyribonucleic acid
TF	transcription factor
TQ	target quadrant
US	unconditioned stimulus
WT	wild type

CHAPTER I:
INTRODUCTION

PREFACE

A remarkable feature of the brain is its ability to process enormous amounts of information subserving the formation and retrieval of memory. Understanding the nature of memory has been a quest for philosophers, psychologists and neurobiologists. However, like many attributes of the mind, memory is only poorly understood.

Memory is not encoded and fixed in the brain like a videotape that can be replayed in a mental VCR to provide accurate recall years after the event took place. Instead, the ability to form, retain and recall memory seems to involve dynamic processes, and depends on numerous factors, including the nature of the information, the circumstances of its acquisition, and the context of its recall. Furthermore, memory, in many cases, is prone to fading with time.

The purpose of the present study was to understand the molecular events occurring in the brain during both the initial formation and the recollection of a memory. The first part of this introduction describes the major concepts, facts and unsolved problems of memory. The second part presents current experimental tools for addressing these questions. Finally, the scope of this thesis will be outlined.

1.1 MEMORY: FORMATION AND REACTIVATION

1.1.1 The neuropsychological perspective

1.1.1.1 A psychological definition of memory

Memory is the retention of, and ability to recall, information, personal experiences and procedures. In humans, memory can be broadly classified as *declarative* and *non-declarative* (Fig. 1.1) (for review see Milner et al., 1998). *Declarative* memory (or *explicit* memory) is what is ordinarily meant by the term memory. It is a memory that we can bring into conscious awareness, reflect upon, and speak about. An example of such memory would be memory for the fact "the United Kingdom is a member state of the European Union". It is propositional, that is to say, it can be either true or false. *Non-declarative* memory (*implicit* memory), on the other hand, "underlies changes in skilled behaviour and the ability to respond appropriately to stimuli through practice, as a result of conditioning or habit learning" (Milner et al., 1998). It is a memory that lies outside conscious awareness (i.e. its recall is unconscious), and for that reason cannot be put into words. In contrast to declarative memory, it is a "know how" or procedural memory, such as knowing how to ride a bicycle. It is expressed through performance, and therefore is neither true nor false.

Declarative memory can be divided into *episodic* memory, memory of events and episodes, and *semantic* memory, memory of facts independent of context (Fig. 1.1). The psychologist who first proposed this idea was Endel Tulving (Tulving, 1972). For episodic memory, the subject not only has the memory, but can remember the setting in which the memory was acquired. It is about "what," "where," and "when" (Clayton and Dickinson, 1998; Nyberg et al., 1996). Episodic memories therefore include autobiographic memories, such as

memories of childhood, and non-personal memories such as public events, films or documentaries. Conversely, for semantic memory, the subject cannot (or does not) recall the context of the initial learning. Semantic memory can therefore be defined as the knowledge for the meaning of words and facts about the world, such as, the capital of Finland, the president of the USA during the Vietnam War, the definition of the word elephant, and knowledge of public events and personalities.

Non-declarative memory can also be sub-divided into different kinds, including procedural (skills and habits), priming, simple classical conditioning and non-associative learning (Fig. 1.1). This type of memory is however not the focus of this thesis. It will therefore not be further described here. For a comprehensive description of non-declarative memory, the reader is referred to *Memory and Brain* by Larry Squire (Squire, 1987a).

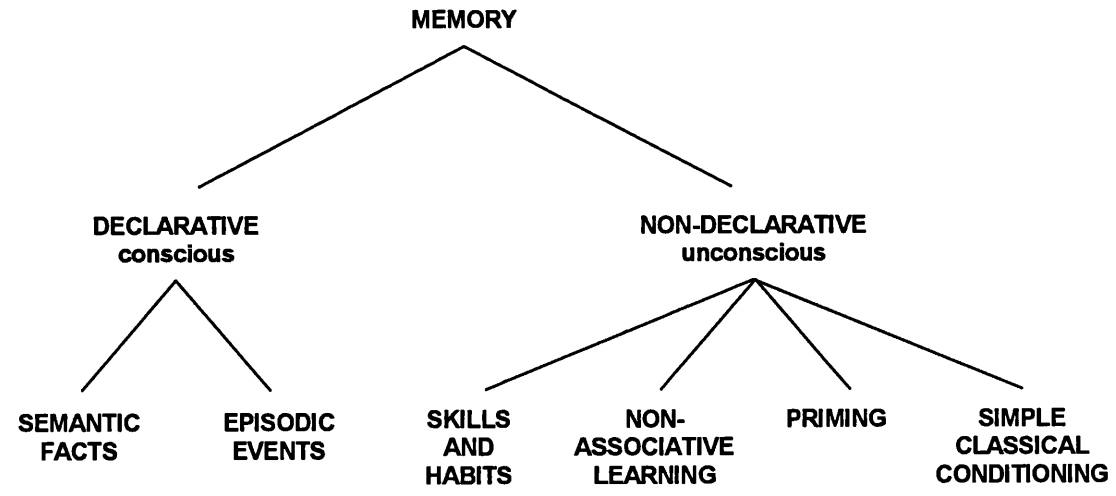

Figure 1.1: **Different types of memory** (Adapted from Squire 1987)

1.1.1.2 Memory consolidation and the stabilisation of a memory trace

The idea that fresh memories are initially rather unstable traces (short-term memory, STM) that need time to stabilise in order to become long lasting and fixed (long-term memory, LTM) emerged from several studies with human subjects. For example, it had been noticed that "the interval of a single night will greatly increase the strength of (...) memory" (Quintillian, reported in First century AD; From Dudai 2004), and that distracting the attention of a subject from newly learned material would impair subsequent recall of that material (e.g. Müller and Pilzecker reported in 1900; from Lechner et al 1999). At the end of the 20th century, the term "consolidation" (from the Latin *consolidare*, to make firm) was introduced to describe the memory phase(s) during which this stabilisation would take place (term attributed to Müller and Pilzecker, 1900).

Experimental evidence showing that this consolidation process was time-dependent came half a century later, with studies showing that treatments that disrupt the normal functioning of the brain, such as electroconvulsive shock (ECS), produced amnesia shortly after learning, but had no effect if applied several hours later (Duncan, 1949;McGaugh, 1966). Numerous studies have since confirmed these findings, and a time-dependent memory consolidation process is now widely accepted as a pre-request for long-term memory.

1.1.1.3 Declarative memory and the role of the hippocampus

Understanding which areas of the brain are important in the consolidation and storage of memory has been of particular interest in the field since the beginning of the 19th century. A major breakthrough came in 1957, when Scoville and Milner reported "the importance of the hippocampal region for normal memory function" (Scoville and Milner, 1957). Bilateral hippocampal lesions suffered as part of a medial temporal-lobe resection in the attempt to

treat epilepsy, schizophrenia or manic depression, resulted in anterograde amnesia, the incapability of making new memory. Included in this study was the now well-known patient H.M (for review see Corkin, 2002).

H.M. underwent the surgical procedure to relieve medically intractable epilepsy on 1st September 1953, at the age of 27. In an examination performed 28 months after the operation, psychologist Brenda Milner described the memory defect of patient H.M. as follows:

> *"The memory defect was immediately apparent. The patient gave the date as March 1953, and his age as 27. Just before coming into the examining room he had been talking to Dr Karl Primbram, yet he had no recollection of this at all and denied that anyone had spoken to him. In conversation, he (...) seemed scarcely to realize that he had had an operation."*

Since Scoville's and Milner's original report, a more thorough investigation of H.M.'s anterograde amnesia has revealed that H.M. is deficient in most forms of episodic and semantic memory tested to date (for review see Corkin, 2002). His memory defect has persisted without improvement to the present time.

Nevertheless, H.M. is capable of very short-term (or immediate) declarative memory (Scoville and Milner, 1957). He is able to retain a three-figure number or a pair of unrelated words for several minutes if undisturbed. However, the instant his attention is diverted to a new topic, H.M. forgets the number or the paired-words to be remembered. It is therefore not the acquisition of the memory that is affected by the lesions to the medial temporal lobe, but rather the capacity to consolidate the newly acquired and fragile memory trace into a longer lasting memory.

Certain classes of memory function are, however, spared. Brenda Milner reported in 1962 that H.M. could learn a mirror drawing task efficiently with stable retention from day to day,

therefore developing a procedural memory, despite the absence of declarative memory for any detail of the testing session (Milner, 1962). Later, it was shown that H.M.'s memory capacities were not limited to skill learning and that other kinds of non-declarative memory, such as habit learning or the phenomenon of priming, were preserved (for review see Corkin, 2002).

Studies of other patients with bilateral lesions of the medial temporal lobe have led to the same conclusions: the damage impairs consolidation of declarative memory, but not that of non-declarative memory (reviewed in Milner et al., 1998; Squire, 1987b; Squire, 1987a; Squire and Zola-Morgan, 1991). Therefore, declarative memory depends on the integrity of the medial temporal lobe, whereas non-declarative memory appears to be supported by different areas of the brain, likely to be the same areas as those responsible for perceiving and analysing the information that is to be remembered.

As soon as the clinical symptoms of H.M. were described in 1957, attempts began to establish animal models of his condition. But how could declarative memory be studied in experimental animals? Indeed, animals cannot declare anything, and are not thought to be capable of conscious recollection. Declarative memory has nevertheless many critical features that can be investigated in other mammals such as mice (Eichenbaum, 1997). Declarative memory is concerned with the ability to remember or recognise people, places and objects. Rodents, for example, can easily be tested about their memories for places, objects and odours. Studies exploring such memories in rodents and other mammals have confirmed that lesions of the medial temporal lobe, and in particular lesions to the hippocampus and related structures, interfere with the consolidation of this type of memory (Zola-Morgan and Squire, 1984, reviewed in Squire, 1992; Squire and Zola, 1998). The behavioural tasks that require the hippocampus have been termed hippocampus-dependent

tasks. Some of these hippocampus-dependent tasks are described in detail later in this introduction.

1.1.1.4 Systems consolidation and long-term storage of hippocampus-dependent memory

The hippocampal formation is important for the consolidation of new episodic memory. Another striking observation of amnesic patients like H.M. is that not only do they suffer from anterograde amnesia, but they also suffer from retrograde amnesia (loss of memory for events and facts that occurred before the brain damage) (Sanders and Warrington, 1971; Scoville and Milner, 1957). Interestingly, in most patients with damage limited to the hippocampal formation, this retrograde amnesia is graded: no recollection of more recent memories, while more remote memories, such as childhood memories, are spared (e.g. (Kapur and Brooks, 1999; Reed and Squire, 1998). Patient H.M., for example, was described in the original report by Scoville and Milner to revert constantly to boyhood events (Scoville and Milner, 1957). H.M.'s retrograde amnesia was later diagnosed to extend back to age 16; 11 years before his operation (Sagar et al., 1985). This type of temporally-graded retrograde amnesia has also be observed in most animal models of human amnesia that have focused on the effect of lesions to the hippocampal formation on memory (e.g. Anagnostaras et al., 1999; Kim and Fanselow, 1992, reviewed by Squire et al., 2001).

These findings have led to the view that the hippocampal formation damaged in amnesia has a temporary role in memory storage, and that the memory initially depends on this structure but become independent as time passes. The memory is thought to reorganise over time. Whether this involves actual transfer of the memory trace out of the hippocampus and storage in other, neocortical brain areas is at present, unclear (Reviewed by Anagnostaras et al., 2001). The process by which the memory becomes independent of the hippocampus has

been termed "systems consolidation". Systems consolidation is a slow process, which may take weeks, months or even years to be accomplished.

The term consolidation is therefore used for two processes. One type is accomplished relatively quickly, and is thought to occur within a single structure of the brain, whereas the other takes longer, and refers to the reorganisation and spread of the hippocampal memory trace out of the hippocampus. To avoid confusion the former process will henceforth be referred to as "cellular consolidation".

Although the idea of systems consolidation is nowadays widely accepted, a proportion of scientists nevertheless deny it, and in contrast, believe that memory never becomes independent of the medial temporal lobe (Nadel and Moscovitch, 1997; Nadel and Moscovitch, 2001). This view is based on clinical observations on an important proportion of amnesic patients with damage to the medial temporal lobe, for whom the retrograde amnesia is ungraded: the patients lack episodic memories for any period of their life. However, thorough investigations of the extent of the brain damage in some of these severely impaired patients suggest that ungraded retrograted amnesia occurs when, in addition to medial temporal lobe damage, there is cortical pathology (Kapur, 1993; Reed and Squire, 1998; Squire and Alvarez, 1995). As systems consolidation predicts that memory might transfer to cortical areas, larger brain damage that extends to the cortex could explain why the amnesia observed in these patients is ungraded (Squire et al., 2001).

1.1.1.5 Memory reactivation and the concept of memory reconsolidation

A new memory thus undergoes a time-dependent consolidation process in order to be maintained. The original consolidation theory predicted that for a given memory, consolidation occurs only once, whether at the cellular or systems level. When consolidated, a memory would be permanent and unmodifiable. However, this idea was challenged in 1968 by Lewis and colleagues (Misanin et al., 1968). ECS given 24 hours after a behavioural task called "fear conditioning" did not generate amnesia in rats, therefore confirming previous findings that the memory was consolidated by that time (Duncan, 1949; McGaugh, 1966). If, nevertheless, the consolidated memory was reactivated immediately before the ECS administration, amnesia was observed the following day. Memory reactivation in the absence of ECS administration did not generate amnesia. Lewis's interpretation of these results was that the reactivation of the consolidated memory transferred it from an inactive stable state to a labile active state, in which it was susceptible to interference. The memory then later returned back to an inactive state, if not interfered with (Misanin et al., 1968). These experiments were soon replicated by many researchers, using pharmacological treatments as well as ECS, and it was demonstrated that the amnesic agent was effective in inducing amnesia only if administered in a short time-window around the time of memory reactivation (e.g. Davis and Hirtzel, 1970; Howard and Meyer, 1971; Lewis et al., 1972; Potts, 1971; Schneider and Sherman, 1968).

Just as for new memories, consolidated memories that are reactivated therefore undergo a time-dependent stabilisation process similar to the initial consolidation process. This stabilisation process has been termed memory "reconsolidation" (Nader et al., 2000; Przybyslawski and Sara, 1997). At any one time, a memory is thus either in an active or an inactive state (Lewis, 1979). An active memory is (i) a newly formed memory that has not

yet consolidated, or (ii) an established retrieved memory that has not yet reconsolidated. An inactive memory is a consolidated or reconsolidated memory (Fig. 1.2).

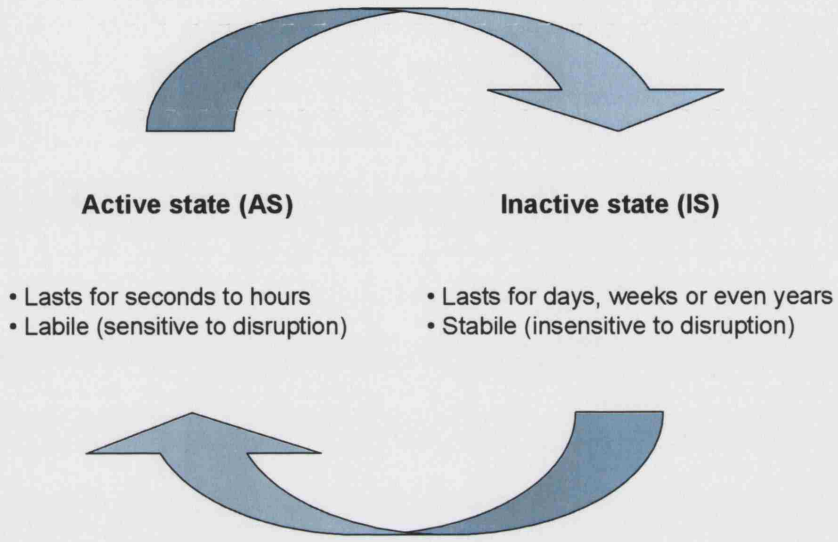

Figure 1.2 Memory model proposed by Lewis (Adapted from Nader 2003).
A new memory is initially in a labile AS and with time enters the IS. Reactivation of a consolidated memory which is in the IS returns it to the AS. With time, this reactivated memory returns once more to the IS.

Information on the brain areas involved in memory retrieval and subsequent reconsolidation is relatively sparse and fragmented. Neuronal models predict that retrieval of information reactivates the same brain regions, or at least some of the brain regions that were engaged during the initial learning and encoding of the information (Nyberg et al., 2000). However, experimental evidence on whether the consolidation of new memory and the reconsolidation of established memory engage the same brain areas is mixed. Whereas some studies have shown that consolidation and reconsolidation occur in the same anatomical structures (e.g. Debiec et al., 2002; Nader et al., 2000), others have suggested that the two processes engage different brain areas (Bahar et al., 2004; Tronel and Sara, 2002). It is therefore likely that the

extent to which same brain structures are involved in consolidation and reconsolidation depend on the nature of the memory in question.

All the studies described here have looked at the reactivation and subsequent reconsolidation of recent memory. As described earlier, initial consolidation also occurs at the systems level, whereby hippocampus-dependent memory becomes hippocampus-independent. Two groups recently investigated, each using a different task, whether a remote memory that had become hippocampus-independent could also undergo reconsolidation. Whereas one of the groups showed that reconsolidation of remote memories does occur and depends on the integrity of the hippocampus (Debiec et al., 2002), the other group failed to demonstrate reconsolidation at the systems level (Milekic and Alberini, 2002). Whether or not a memory undergoes systems reconsolidation thus seems to depend on the type of memory under investigation.

Consolidation and reconsolidation might engage the same brain structures, and hippocampus dependent memory might undergo systems reconsolidation. It is however unclear whether the cellular and molecular mechanisms underlying the two processes are the same. In the next section, an overview of the neurobiological mechanisms underlying memory consolidation will be presented. Then the similarities with mechanisms underlying reconsolidation will be discussed.

1.1.2 The neurobiological perspective

In addition to understanding the systems problem of memory, research has also focused on understanding *how* memory is formed, maintained and expressed, in other words, what are the cellular and molecular bases of memory, and what processes are involved in memory consolidation and reconsolidation. This is the neurobiological problem of memory. Most studies of this kind use animal models.

1.1.2.1 Memory consolidation at the cellular and molecular level

Long lasting changes in synaptic efficacy as a mechanism underlying memory

By the end of the nineteenth century, neurons were suspected to form networks and have points of connection, at which they would communicate with each other. Sherrington and Verrall called these points "synapses" (from Greek: "syn" meaning "together" and "haptein" meaning "to clasp"). An idea of how memory could be sustained was proposed by the Spanish neuroanatomist Santiago Ramon y Cajal in 1894. The formation of long-term memory would require changes in synaptic connectivity. "Existing neurons would grow more branches and strengthen their connections with other neurons so as to be able to communicate with them more efficiently" (Ramon y Cajal reported in 1894; from Milner et al, 1998). This idea, although overlooked when first introduced, was further developed by Donald Hebb in 1949. Hebb postulated that:

> *"When an axon of cell A (...) excite(s) cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased"* (Hebb, 1949).

Thus, the synaptic connection between two neurons would be strengthened when the neurons were active at the same time.

This postulate was verified experimentally decades later with the description of long-term potentiation (LTP)(Bliss and Lomo, 1973). Bliss and Lømo demonstrated that brief trains of high frequency stimulation to excitatory neurons led to long lasting increase in synaptic efficacy. This phenomenon they termed LTP. LTP gained a lot of interest in the memory field, since it was history-dependent, enduring, and had the primary properties required for memory: *cooperativity*, *associativity* and *input-specificity* (Bliss and Collingridge, 1993). *Cooperativity* describes the necessity for coincident activity of two or more neurons, in other words the requirement for a level of activity above a certain threshold in order for LTP to be triggered. *Associativity* describes the timing relationship between the activity of the pre- and postsynaptic terminals whereby LTP is only triggered if the activity of the two terminals of the synapse is close enough in time. Associativity therefore enables the phenomenon whereby a "weak" neuronal input that converges onto the same neuron as a separate "strong" input can be potentiated, if both inputs are active at the same time. *Input-specificity* implies that other inputs that were not active at the time of the tetanus are not potentiated. Therefore LTP generated at one synapse does not spread to other synapses in the same cell.

An understanding of one molecular mechanism underlying the induction of LTP came with the discovery of the N-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamate receptor occurring on the post-synaptic membrane. It was demonstrated that high frequency stimulation led to NMDA receptor activation, and that the receptor was essential for the induction of LTP at certain CA1 synapses (Harris et al., 1984; Herron et al., 1985). The NMDA receptor is fully functional only when the two neurons forming the synapses at which it is present are active at the same time; thus it behaves as a molecular coincidence-detector. For the channel to open, and LTP to be induced, two events have to occur simultaneously: (i) the neuron of the pre-synaptic terminal must be activated so that glutamate is released from that terminal and can bind to the NMDA receptor on the

postsynaptic membrane, and (ii) the postsynaptic terminal must be sufficiently depolarised, so that a voltage-dependent block, a Mg^{2+} ion present in the channel, is relieved (Fig. 1.3).

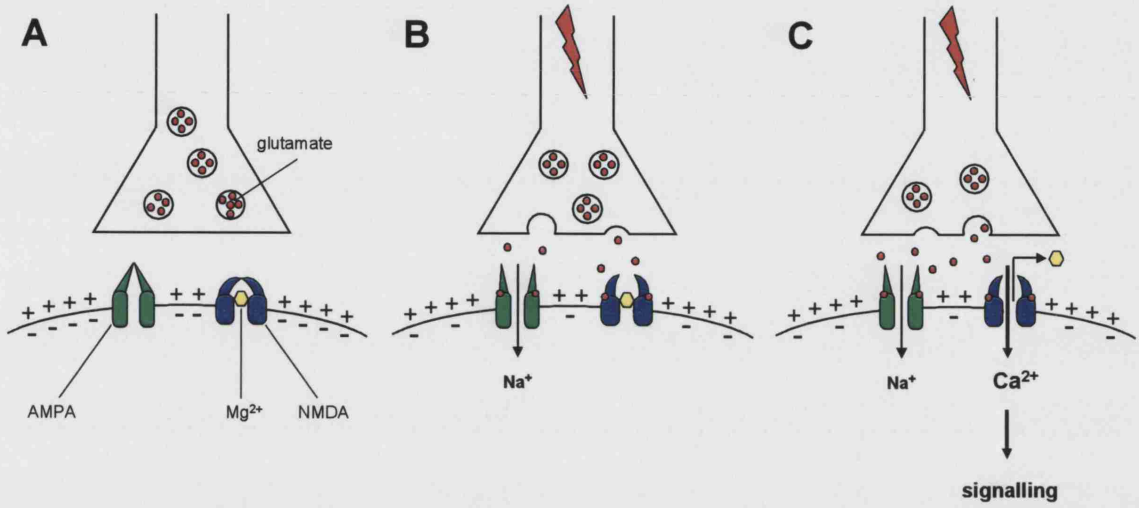

Figure 1.3 NMDA receptor activation and LTP induction at the synapse
(A) synapse at rest. Vesicles containing glutamate are present at the presynatpic terminal. The potential of the postsynaptic membrane is -60mV. The AMPA and NMDA on the post-synaptic membrane are inactive, as there is no glutamate in the synaptic cleft.
(B) An action potential arrives at the presynaptic terminal resulting in the fusion of the presynaptic vesicles with the membrane and release of glutamate into the synaptic cleft. The glutamate binds to the AMPA receptors, opening the channel and allowing Na^+ ions to flux into the postsynaptic cell. Glutamate also binds to the NMDA receptor, opening the receptor channel, but a Mg2+ ion present in the channel prevents channel activity.
(C) As more Na^+ ions enter the postsynaptic cell, the membrane becomes more depolarised. Once the membrane is sufficiently depolarised, the Mg^{2+} block of the NMDA receptor is relieved. This activates the NMDA receptor and allows Ca^{2+} to flux into the postsynaptic cell. The Ca^{2+} in turn activates downstream signalling cascades presumably resulting in LTP. It is not known how these cascades affect the properties of the synapse to accomplish LTP.

Depolarisation of the postsynaptic cell is achieved through the activation by glutamate of a second type of ionotropic receptor on the postsynaptic membrane, the α-amino-3-hydroxy-5-methyl-4-isooxazoleproprionicacid (AMPA) receptor, although back-propagating action potentials in the postsynaptic neuron may also contribute to depolarisation (Colbert, 2001). The activated AMPA receptor allows Na^+-flux into the postsynaptic cell leading to depolarisation of the postsynaptic membrane, or excitatory post-synaptic potential (EPSP).

Repetitive tetanic stimulation of the pre-synaptic pathways results in recurrent AMPA receptor activation and sufficient postsynaptic depolarisation for the removal of the voltage-dependent Mg^{2+} block from the NMDA receptor (Fig 1.3). The activated NMDA receptor allows Ca^{2+} to enter the postsynaptic cell, which trigger signalling pathways that induce LTP. Once the LTP is established and thus the synapse is potentiated, subsequent activation of the presynaptic cell leads to an increased postsynaptic response (Reviewed by Bliss and Collingridge, 1993).

The first suggestion that LTP and the NMDA receptor might be important for memory consolidation was provided by Richard Morris and colleagues who demonstrated that antagonising the NMDA receptor impaired both LTP and spatial learning (Morris et al., 1986). Subsequent studies have shown that treatments (both pharmaceutical and genetic), which impair LTP also often impair LTM formation (for review, see Lynch, 2004). However, to date, all the studies implicating LTP as a L&M mechanism have been correlative. To establish a direct link between LTP and memory, one would require a demonstration that LTP is induced in the brain after a L&M task, and that blocking learning induced LTP blocks LTM formation. Although such a link is still missing, LTP is nevertheless one of the most promising candidate mechanisms underlying behavioural memory.

The NMDA receptor is therefore critical for triggering a form of LTP as well as the cellular events leading to memory consolidation. But how does Ca^{2+} influx through the NMDA receptor trigger the subsequent processes underlying memory formation and storage? What are the molecules involved in changing synaptic efficacy and how are these changes achieved? Some progress in understanding these events has been made in the recent years and will be discussed in the next section.

Memory consolidation requires de novo protein synthesis and transcription

Importance of protein synthesis and transcription for LTM formation

Insight into the way that morphological and physiological changes could be sustained at the molecular level came with the discovery that protein synthesis (translation) is a prerequisite for LTM formation. Initially demonstrated in mice (Flexner et al., 1963), in which treatment with the protein synthesis inhibitor puromycin impaired LTM formation, the requirement for protein synthesis has since being shown in a variety of model systems (reviewed by Davis and Squire, 1984). Protein synthesis has also been shown to be essential for LTP (Frey et al., 1988; Krug et al., 1984). The newly synthesised proteins are thought to influence synaptic function and connectivity.

Although the cell body is classically considered as the site of protein synthesis, research investigating protein synthesis in central nervous system neurons has shown that translation also takes place in dendrites (Schuman, 1999; Steward and Schuman, 2001). Components of the translational machinery are found beneath dendritic postsynaptic sites (Steward, 1983; Steward and Fass, 1983; Steward and Levy, 1982). This synapse-specific location has prompted the following ideas: (*a*) this machinery might synthesise key molecular constituents of the synapse, (*b*) translation in the dendrites might be regulated by activity at the individual postsynaptic sites, (c) local protein synthesis could be a mechanism whereby input specificity of synaptic plasticity is achieved, and (d) dendritic translation might be key to memory consolidation (Schuman, 1999). Consistent with this idea, disruption of the dendritic translation of αCaMKII has recently been shown to impair both synaptic plasticity and memory consolidation (Miller et al., 2002).

Newly synthesised proteins most commonly result from concurrent gene expression, although translation from constitutively present mRNAs probably also occur (discussed in Steward and Schuman, 2001). Shortly after the discovery of the importance of protein synthesis for LTM formation, research intensified in an effort to understand whether transcription (the synthesis of RNA) was also essential. Many studies using both vertebrates and invertebrates emerged, in which inhibitors of RNA synthesis effectively blocked LTM without alteration of STM (e.g. Agranoff et al., 1967; Appel, 1965; Barondes, 1970; Barondes and Jarvik, 1964). However, the results of such studies have to be considered carefully with regard to the role of transcription in LTM formation as the drugs used have substantial side effects, including neurotoxicity (e.g. Nakajima, 1969; Wetzel et al., 1976).

Evidence for a critical role for transcription in memory consolidation has nevertheless come in more recent years from studies making use of genetically modified mice as well as from expression studies. Thus, the CREB protein (cAMP response element binding protein), a major transcriptional "switch" which induces gene expression, has been shown to be important for the consolidation of many types of memory (e.g. Bourtchuladze et al., 1994; Kida et al., 2002; although see Gass et al., 1998; Graves et al., 2002). In addition, changes in gene expression have been observed shortly after the acquisition of many memory tasks (e.g. Hall et al., 2000), and blocking such changes has been shown to impair LTM formation (e.g. Lee et al., 2004). Finally, synaptic activation has been reported to trigger the trafficking of new mRNA transcripts from the soma to the synapses (Steward et al., 1998), and blocking the transport of at least one of these mRNAs, that for αCaMKII, results in impaired synaptic plasticity and memory consolidation (Miller et al., 2002). Therefore, transcription is now widely accepted as a pre-request for memory consolidation.

A Role for CREB mediated transcription

Studies in *Aplysia*, *Drosophila*, and mice have indicated that CREB-dependent transcription is crucial to the consolidation of various types of memory (Bartsch et al., 1995; Bourtchuladze et al., 1994; Impey et al., 1998; Kida et al., 2002; Kogan et al., 1997; Yin et al., 1994; Yin et al., 1995; for additional reviews see Silva et al., 1998; Yin et al., 1994; Yin and Tully, 1996). These studies suggest that particular transcription factors might play an essential role in memory consolidation.

The transcription factor CREB is activated by a variety of extracellular stimuli, and induces the expression of genes with such diverse functions as cell survival, cell growth and cell differentiation. In neurons, in addition to its prominent role in LTM formation, CREB has been shown to be involved in stress, growth and survival, neuroprotection and circadian entrainment (Reviewed in Lonze and Ginty, 2002). CREB belongs to the bZIP superfamily of transcription factors (TFs), and together with the TFs ATF-1 and CREM-1 forms the CREB family of TFs. CREB family members activate transcription by binding to the CRE-element (cAMP response element) on the DNA. The CRE-element contains the palindromic consensus sequence TGACGTCA. And although many CREB binding sites consist of variations of this consensus motif, almost all have the core sequence CGTCA. CREB is activated by phosphorylation, which induces a conformational change in the protein, exposing the binding site for the CREB binding protein (CBP). Interaction of CREB with CBP results in transcription (Reviewed in Barondes, 1970; Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999).

Learning that subsequently results in LTM formation triggers CREB phosphorylation and CRE-mediated transcription in brain areas important for LTM (Impey et al., 1998; Peters et al., 2003; Wei et al., 2002). For example, hippocampus-dependent L&M tasks induce the

hippocampal expression of BDNF, zif268 and C/EBP, all of which have the CRE-element in their promoter (Hall et al., 2000; Taubenfeld et al., 2001).

The signal-transduction pathways that lead to CREB-mediated transcription during memory consolidation have been studied in both vertebrates (mice and rats) and invertebrates (*Aplysia*, *Drosophila*). The activation of postsynaptic receptors after neurotransmitter release results in the depolarisation of the post-synaptic neuron. Sufficient depolarisation leads to an increase in the concentration of intracellular mediators, such as Ca^{2+} and cAMP. These intracellular mediators activate two major downstream signalling cascades: the cAMP/PKA mediated cascade (triggered by an increase in cAMP levels), and the Ca^{2+}/Calmodulin Kinase mediated pathway (triggered by an increase in Ca^{2+} levels). Activation of these cascades ultimately leads to the activation of CREB-mediated transcription (Fig. 1.4).

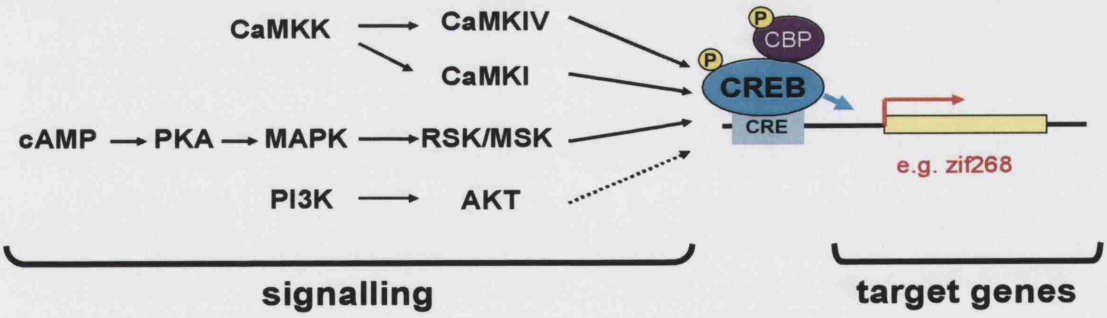

Figure 1.4 Activation of CREB and CREB-mediated transcription
Two major cascades activate CREB during LTM formation: the CaMK pathway and the PKA/MAPK pathway. Other pathways may also activate CREB (e.g. the PI3K pathway)

The increase in intracellular Ca^{2+} in the postsynaptic cell results from extracellular Ca^{2+} influx through the activated neurotransmitter-gated channels, such the NMDA receptors, and through voltage gated L-type channels. The Ca^{2+} entry through these channels is essential for CREB mediated gene expression and LTM formation (Deisseroth et al., 1996;

Hardingham and Bading, 1999; Impey et al., 1996; Kim et al., 1992; Morris et al., 1986). The Ca^{2+} binds to Calmodulin (CaM). CaM in turn activates Ca^{2+}/CaM dependent protein kinase cascade (CaMKs). Of these kinases, CaMKKβ and CaMKIV have been shown to be essential for long term memory formation and CREB activation in the hippocampus (Peters et al., 2003; Kang et al., 2001;Wei et al., 2002; reviewed by Bito et al., 1997; Lonze and Ginty, 2002).

In addition to increasing intracellular Ca^{2+} levels, neurotransmitter release also results in the activation of Adenylate cyclase, an enzyme that synthesises cAMP from ATP. This activation can be mediated through signalling via G-protein coupled receptors (e.g. dopamine receptor) or through increased intracellular Ca^{2+} levels (via Ca^{2+}/Calmodulin). The increase in cAMP levels results in the activation of PKA, important for CREB activation (Impey et al., 1996) and long-term memory formation in both vertebrates and invertebrates (Abel et al., 1997; Kaang et al., 1993; Kandel and Schwartz, 1982; Li et al., 1996).

Although CREB mediated transcription and the pathways leading to CREB activation have been shown to be essential for long term memory formation, one should nevertheless bear in mind that other transcriptional switches might be important for memory consolidation. Indeed, there is growing evidence that other major transcriptional switches, such as C/EBPβ, Elk-1/SRF and the NF-kB family have an important role in LTM formation (e.g. Taubenfeld et al., 2001; Cammarota et al., 2000; Chen et al., 2003; Levenson et al., 2004).

Effector genes of memory consolidation

Many studies have characterised the requirement for *de novo* mRNA and protein synthesis during memory consolidation. However, only a few recent studies have identified the

regulated genes. These genes most likely encode for the effectors that bring about the morphological and physiological changes resulting in increased synaptic efficacy, thought to underlie memory. Expression profiling studies have shown that the regulated genes have very diverse functions and are involved in processes such as regulation of transcription, signalling, growth, metabolism, extracellular matrix, and cell structure (Cavallaro et al., 2002; Leil et al., 2002; Levenson et al., 2004; Stork et al., 2001). A role in LTM memory formation has only been demonstrated for some of the genes. These encode transcription factors, such as the immediate-early genes (IEGs) zif268, BDNF, c-fos and C/EBP (Jones et al 2001; Chen et al., 2003; Fleischmann et al., 2003; Hall et al., 2000; Taubenfeld et al., 2001; Yukawa et al., 1998). Importantly, a role in LTM formation for other types of regulated gene, such as those involved in growth and cell structure, has yet to be demonstrated. One recent study, however, has addressed this issue in an indirect way. The authors, after identifying such effector genes (i.e. not transcription factors) regulated during LTM formation in the mouse, investigated whether these genes were associated with either mental retardation or cognitive impairment (Levenson et al., 2004). They showed that a subset of these genes had indeed been associated with derangement. Thus, although direct evidence for the role of these proteins has yet to be demonstrated, it is certainly the case that the genes regulated during LTM formation are important in normal memory function and understanding their role may help us understand the mechanisms underlying memory and dysfunction in disease.

Memory consolidation and the autophosphorylation of αCaMKII.

In addition to *de novo* protein synthesis and transcription, other proteins, not thought to be involved in the signalling cascades leading to changes in gene expression, are nevertheless important in LTM. One such protein is the αCaMKII, which is abundant in the hippocampus, neocortex, and basal ganglia.

αCaMKII constitutes 1.4% of the total protein mass of the hippocampus (Hanson and Schulman, 1992). It is highly concentrated in excitatory synapses (Kennedy et al., 1990; Miller and Kennedy, 1985), but is also found at lower densities in the cell body (Ouyang et al., 1997; Ouyang et al., 1999). The protein has a remarkable property: it can act as a "molecular memory device", switching to an autonomous active state when exposed to Ca^{2+}/CaM and then remaining active even after the Ca^{2+} is withdrawn (Hanson and Schulman, 1992). Therefore, the activity can persist in the absence of any synaptic input. This permanent, autonomous active state is achieved by a process called autophosphorylation, whereby the kinase phosphorylates itself. Because the autonomous activity of αCaMKII can serve as a molecular memory trace of a prior synaptic input, it has been suggested that the protein could act as a molecular substrate of behavioural memory (Lisman et al., 2002; Lisman, 2003).

Consistent with this idea, hippocampal LTP is associated with autophosphorylation (Barria et al., 1997; Ouyang et al., 1997) and increased activity of αCaMKII (Fukunaga et al., 1993). Similarly, autophosphorylation of αCamKII is increased in the hippocampus during the consolidation of hippocampus-dependent fear memory (Atkins et al., 1998). Furthermore, $\alpha CaMKII^{T286A}$ mutant mice –in which a targeted point mutation prevents the autophosphorylation and therefore the autonomously active form of the protein (Giese et al., 1998)– are impaired in hippocampal LTP (Giese et al., 1998), and have unstable hippocampal place cells (Cho et al., 1998), which are thought to be important for the formation of spatial representations and therefore spatial memory (O'Keefe and Nadel, 1978). In addition, the mutants are impaired in hippocampus-dependent L&M (Carvalho et al., 2001; Giese et al., 1998; Need and Giese, 2003; E.E. Irvine, J. Vernon and K.P. Giese, unpublished results).

Therefore, αCaMKII plays an important role in LTM formation. αCaMKII may accomplish this function by acting as a synaptic "tag", labelling activated synapses, so as to recruit to those synapses macromolecules, including newly synthesised transcripts and proteins, which would in turn facilitate information storage by altering the architecture and physiology of the synapse. For example, it is though that CaMKII participates in the trafficking and anchoring of AMPA receptors at activated synapses, a process thought to be important for increasing synaptic efficacy (Lisman et al., 2002). Activated αCaMKII may also phosphorylate components of the synaptic translational machinery so as to endow activated synapses with the capability to independently control synaptic strength through the local synthesis of proteins. Therefore autonomous activity of αCaMKII specifically at activated synapses may be a mechanism by which *input-specificity* is achieved.

Molecular mechanisms underlying systems level consolidation

To date, most experimental work on the biological processes thought to underlie hippocampus-dependent long-term memory has concentrated on the processes occurring within the hippocampal formation. With time, however, these memories become hippocampus-independent, a process that is thought to involve a transfer of the memory trace out of the hippocampus (see section 1.1.1.4). Little has been done on the interaction between the hippocampal formation and adjacent cortical areas. It is nevertheless becoming increasingly recognised that the cellular and molecular mechanisms occurring in the cortex during systems consolidation and those underlying putative hippocampus-cortex interactions hold at least one of the keys to understanding how memories ultimately come to be represented in the brain. Recently, the first study investigating systems level consolidation at the molecular level showed that αCaMKII activity in the neocortex is essential for the establishment of permanent hippocampus-dependent memory (Frankland et al., 2001;

Frankland et al., 2004). More studies of this kind are required in order to better understand systems level consolidation.

1.1.2.2 Memory reconsolidation at the cellular and molecular level

As described in section 1.1.1.5, the reactivation of a consolidated memory can return it to a labile state, and induce a second consolidation process, termed reconsolidation (Nader et al., 2000; Sara, 2000). Like the initial learning, such memory reactivation has been shown to induce *de novo* transcription (Hall et al., 2001; Strekalova et al., 2003). Inhibition of transcription or protein synthesis within a short time window after the recall impairs the previously consolidated memory (Anokhin et al., 2002; Debiec et al., 2002; Kida et al., 2002; Milekic and Alberini, 2002; Taubenfeld et al., 2001). Thus, both consolidation and reconsolidation require transcription and protein synthesis. But are the mechanisms underlying the two processes otherwise similar?

Only a few molecular studies have compared memory consolidation and reconsolidation. An early study implied that consolidation and reconsolidation engage different molecular mechanisms, as the transcription factor C/EBPβ is required in the hippocampus for consolidation but not reconsolidation of memory in a passive avoidance task (Taubenfeld et al., 2001). However, this study failed to show requirement for the hippocampal structure in reconsolidation in this task. Since consolidation and reconsolidation may engage different anatomical structures (Bahar et al., 2004; Tronel and Sara, 2002), the transcriptional difference observed in the hippocampus by Taubenfeld et al. is difficult to interpret in terms of molecular mechanisms shared by consolidation and reconsolidation. In contrast more recent studies suggest that reconsolidation is a recapitulation of consolidation at the molecular level. Both consolidation and reconsolidation of object recognition memory induce activation of the mitogen-activated protein kinase in the hippocampus (Kelly et al.,

2003). Furthermore, the expression of the immediate-early genes (IEG) c-fos and zif268 is up-regulated in the hippocampus during consolidation and reconsolidation of contextual fear memory (Hall et al., 2000; Hall et al., 2001; Stanciu et al., 2001; Strekalova et al., 2003). Additionally, zif268 is required for both consolidation and reconsolidation of object recognition memory (Bozon et al., 2003; Jones et al., 2001). However, it would be over interpreting these results to state that reconsolidation is the same process as consolidation. And indeed, a recent study has demonstrated for the first time a double dissociation between consolidation and reconsolidation (Lee et al., 2004). Lee and colleagues showed that consolidation and reconsolidation are mediated by independent mechanisms; BDNF is important for consolidation but not reconsolidation, whereas Zif268 is important for reconsolidation, but not consolidation. Although this study demonstrated that consolidation and reconsolidation differ, it is still unclear whether consolidation and reconsolidation are mediated by completely different molecules or whether there are overlapping uses of molecules but deployed different ways by the two processes. A systematic comparison of the mechanisms triggered during consolidation and reconsolidation, for example at the transcriptional level, may clarify this issue.

Although reconsolidation at the systems level has been demonstrated, and is dependent on protein synthesis in the hippocampus, the underlying cellular mechanisms are unknown. Again, a transcriptional comparison of the processes involved in cellular and systems reconsolidation could illuminate the question.

1.2 EXPERIMENTAL APPROACHES TO STUDYING MEMORY

1.2.1 Mouse behaviour and the study of memory

Animal models for studying memory are popular as they provide many advantages over studies involving human subjects. Most of the data from humans has come from the study of amnesic patients. Although such studies are very informative, it is nevertheless the case that studies in patients necessarily depend on retrospective methods. As a result, it is difficult to sample memory evenly across time periods preceding the morbid event and hard to compare performance scores across time periods. Furthermore, studying the cellular and molecular mechanisms underlying memory consolidation in humans is inconvenient as it relies on naturally occurring mutations, polymorphisms or molecular dysfunctions.

In contrast, animal studies allow better control, with respect to the timing and strength of original learning (as they do not rely on retrospective results), and the extent of the lesions sustained, which can be rigorously controlled and verified by autopsy. Furthermore, studies of molecular and cellular processes are more feasible in animals: tissue extraction can be performed shortly after learning, invasive drugs can be used, and genetic manipulation is possible (see below).

Here, the advantages of using mice for the study of memory will be outlined, and the behavioural tasks developed to study memory in rodents will be discussed with an emphasis on the tasks used in this thesis.

1.2.1.1 The mouse model in learning and memory studies

Rodents, and in particular mice, have become a popular animal models to study learning and memory, for several reasons. First, they are easy and relatively cheap to maintain and breed. They have a short generation time (the gestation is 19.5 days for mice) and produce large litters (usually 6-10 pups for mice).

Second, rodents, and in particular mice, perform well in a variety of behavioural tasks that test memory functions similar to those of both declarative (i.e. memory for space, objects and odours) and non-declarative memory.

Third, the anatomy and the intrinsic circuitries of the hippocampus proper and related structures are relatively well conserved between humans and rodents. For both, the hippocampal formation comprises three main regions: (i) the Ammon's horn (*cornu Ammonis*, which is divided into CA1, CA2 and CA3), the dentate gyrus (DG) and the subiculum (Fig. 1.5). The hippocampus comprises a *trisynaptic circuit*, in which (i) the granule cells of the DG receive input from the entorhinal cortex via the perforant pathway, (ii), the axons of the DG granule cells project onto the pyramidal cells of area CA3 (the mossy fibre pathway), (iii) the axons of the CA3 pyramidal cells project onto the pyramidal cell of area CA1 (the Schaffer collateral pathway) (Fig. 1.5). Sensory signals from the entorhinal cortex enter the hippocampus via two main pathways (Steward and Scoville, 1976): (i) via the perforant path, which projects onto the granule cells of the DG, and from which the signals are then processed sequentially in the hippocampal CA fields before returning to the entorhinal cortex via CA1 pyramidal neuron, and (ii) via the temporoammonic (TA) path, which project directly onto CA1 neurons and modulates plasticity and spiking in CA1 neurons, and therefore the output of the hippocampal formation (Remondes and Schuman, 2002).

Fourth, genetic manipulation is possible in the mouse. In the 80's and 90's, methods whereby the genome of the mouse could be altered were developed, making it possible to add, delete or slightly modify genes and therefore the corresponding proteins (Giese, 1999; Jaenisch, 1988; Mansour, 1990; Wehner et al., 1996). This allows one to study the role of specific molecules in learning and memory, across the whole lifespan of a mouse.

Finally, the genome of the mouse has been sequenced. This not only facilitates the genetic manipulation of mice, but also allows a more in-depth study of genes that are involved in learning and memory. The genome project has allowed, for example, the development of microarrays, a technology described later in this thesis, which allows one to scan the whole mouse genome for gene expression changes. The accessibility to the mouse genome has also made it possible to identify gene families and genes sharing common regulatory sequences.

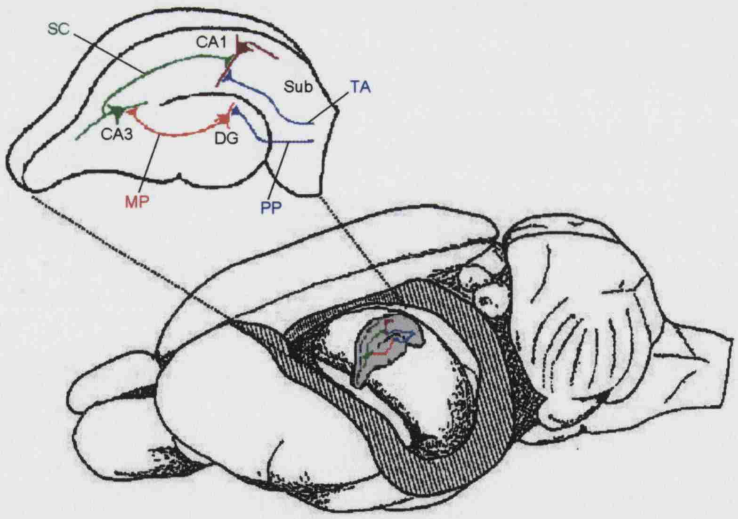

Figure 1.5 A sagital view of the rodent hippocampus (Adapted from Amaral and Witter, 1989)
PP: perforant path; MP: mosy fibre path; SC: schaffer collateral path; TA: temporoammonic path
Sensory signals from the entorhinal cortex enter the hippocampus via two main pathways: the PP and the TA.

1.2.1.2 Single vs. multiple trial learning tasks

Memories are not all acquired with the same ease. Some memories are acquired instantly whereas others require training. For example, a memory for an emotional or traumatic event, such as the death of a loved one, will be acquired instantaneously, and will be retained for life (unless damage occur to the relevant brain structures, as in the case of patient H.M.). In contrast, learning a language or the precise dates of a historical event will usually require several "training" sessions, and yet, such memories will be very easily prone to forgetting, if not constantly used.

Such a distinction can also be made for rodents, and the tasks that are used to study these different memories have been named accordingly. Thus, memory tasks that will require only one training session are called single-trial L&M tasks. By contrasts, tasks which require several training sessions are called multiple-trial incremental L&M tasks.

Single-trial L&M tasks can be categorised into either (i) associative Pavlovian conditioning tasks or (ii) tasks that make use of instinctive behaviours. In case of the former, the animal associates a neutral stimulus (conditioned stimulus, CS), for example a sound, with an aversive stimulus, (unconditioned stimulus, US), for example an electric shock (Dickinson and Mackintosh, 1978). Such tasks include the passive avoidance task (Stubley-Weatherly et al., 1996), conditioned taste aversion (Yamamoto et al., 1994, 1995), and contextual and cued fear conditioning (Kim et al., 1993; Phillips and LeDoux, 1992). Single trial learning tasks that make use of instinctive behaviour include the object recognition task (Hammond et al., 2004) and the social transmission of food preference task (Clark et al., 2002). Single trial L&M tasks are particularly attractive for studying memory. The acquisition of the task can be temporally dissociated from the consolidation of the memory for the task, since the consolidation of the memory is induced by a single training session. Single trial learning

tasks are therefore ideal for understanding the molecular mechanisms underlying memory consolidation.

Multiple-trial incremental L&M tasks can be broadly categorised into spatial and non-spatial tasks. As several training-sessions are required, it is difficult to dissociate the learning (acquisition) from the consolidation of the memory. Therefore, it is difficult to find a time point at which the molecular mechanisms observed would be purely involved in memory consolidation and not learning. The non-spatial tasks are associative conditioning tasks. An example of such tasks is eye-blink conditioning, where the subject learns to associate a tone (CS) with a shock or puff of air to the eye lid (US) (Attwell et al., 2002; Kim and Thompson, 1997). In spatial multiple-trial learning tasks, the animal has to develop a memory for place using cues present in the training environment to navigate through a maze. Examples of such tasks include some versions of the radial arm maze and the Morris water maze (Hodges, 1996; Morris, 1984; Olton and Samuelson, 1976).

1.2.1.3 Memory tasks used in the present study

Behavioural memory tasks can be further categorised into hippocampus-dependent and hippocampus-independent, on the basis of whether of not they require the integrity of the hippocampus in order to be performed. In this thesis, two hippocampus-dependent tasks were used –contextual fear conditioning (single-trial) and Morris water maze (multiple-trial) – and one hippocampus-independent task was used –cued fear conditioning. These tasks are described here.

Contextual and cued fear conditioning

Fear conditioning is a single trial learning task which exploits a form of associative learning found in many species (Dickinson and Mackintosh, 1978). Animals learn to "fear" a previously neutral stimulus (CS) simply because of its temporal association with an aversive stimulus (US), a foot-shock. Conditioned animals, when exposed to the CS, exhibit an adaptive and species-specific behavioural response called "freezing", during which the animal tends to refrain from all but respiratory movement (Fanselow and Bolles, 1979; Miller and Weiss, 1969).

Two main types of CS are commonly used to study fear memory: context and cue. For contextual conditioning, the mouse is placed in a novel environment (the context, CS) and receives a foot-shock (US). The animal makes an association between the context and the shock and will freeze the next time it is returned into that context. For cued conditioning, a tone (CS) is played and co-terminates with the foot-shock (US). The animal makes an association between the tone and the shock and will freeze the next time it is presented with the tone. Unlike cued conditioning, which does not require the activity of the hippocampus, contextual conditioning is dependent on the activity of the hippocampus and the amygdala (Kim et al., 1993; Kim and Fanselow, 1992; Phillips and LeDoux, 1992), and thus can be used to study hippocampus dependent memory consolidation.

During contextual fear memory consolidation, two memory processes are thought to occur: (i) the construction of a conjunctive representation of the independent features of the environment (or context), and (ii) the formation of a context-US association (Rudy, 1996; Rudy and Morledge, 1994; Young et al., 1994). This idea has emerged from experiments in which a *context-pre-exposure facilitation effect* (CPFE) has been observed. In the classical contextual conditioning paradigm, the animal is allowed to explore the environment for 1

min or more before the shock is delivered. Fanselow reported that if the animal is shocked immediately after being placed in the chamber, conditioning was poor (immediate shock effect) (Fanselow, 1990), suggesting that a representation for the context must be formed before the association can take place. Consistent with this idea, the level of freezing could be significantly enhanced by pre-exposing the animal to the context the day before conditioning. Pre-exposure to the context must therefore provide the animal with a representation of the environment, which can then be retrieved and associated with the shock during the immediate shock experiment. Since Fanselow's original experiment, CPFE effect has been described by other groups (e.g. Westbrook et al., 1994, for review see O'Reilly and Rudy, 2001).

There is evidence that the hippocampus plays a critical role in the storage of the memory for the contextual representation, as hippocampal protein synthesis inhibition and lesions impair CPFE (Barrientos et al., 2002;Rudy et al., 2002). The amygdala on the other hand, does not appear to be important, as inhibition of protein synthesis in the amygdala has no effect on CPFE (Huff and Rudy, 2004). There is controversy, however, as to where the context-US association is generated and stored. Some researchers believe that the amygdala is the site for associating CS with shock (for a review, see Fanselow and LeDoux, 1999), whereas others think that emotionally arousing associative memories are stored in extra-amygdalar regions, but that the amygdala modulates the strength of these memories (Cahill and McGaugh, 1998; Lehmann et al., 2000; McGaugh, 2004).

As in human declarative memory, contextual memory in rodents is initially dependent on the hippocampus, but becomes independent of that structure with time. Thus lesions one day after conditioning abolish contextual fear in rats, whereas lesions 28 days after conditioning have no effect (Kim and Fanselow, 1992).

The molecular mechanisms underlying contextual memory consolidation have been extensively studied. Conditioning induces the phosphorylation and thereby the activation of CREB in the hippocampus (Wei et al., 2002), resulting in CRE-mediated transcription (Impey et al., 1998). Consistent with this idea, contextual and cued fear memory is dependent on CRE-mediated transcription (Kida et al., 2002). A large amount of genes regulated during contextual memory consolidation have been identified.

The phenomenon of reconsolidation has been most extensively studied in fear conditioning. Reconsolidation of cued memory requires the amygdala (Nader et al., 2000) and that of contextual memory the hippocampus (Debiec et al., 2002). As consolidation and reconsolidation of contextual fear memory both depend on the integrity of the hippocampus, contextual memory consolidation is therefore an ideal task to compare the molecular events underlying both processes. Recently, it was demonstrated that systems reconsolidation of contextual fear memory occurs (Debiec et al., 2002), and that remote contextual memories are more stable than recent memories, requiring a longer reactivation session in order to become sensitive to disruption by anisomycin (Suzuki et al., 2004).

The Morris Water Maze task

The Morris Water Maze (MWM) is a multiple trial incremental L&M task. It was developed by Richard Morris in 1981 (Morris, 1984). The task tests for spatial memory, or memory for place (Morris, 2001). In this task, the rodent needs to use distal spatial cues to successfully navigate its way within a pool to a hidden escape platform.

Usually, the animal first develops a non-spatial strategy to find the platform. For example, it may swim at a particular distance from the pool wall. But through practice, the animal develops a spatial strategy, using the distal cues available, and will navigate relatively

directly to the platform location from any starting point. One can test for such spatial memory by removing the platform; this is called a "probe trial". Many different measures can be used during the probe trial to determine whether the animal uses spatial cues to find the platform. One of them consists of dividing the pool area into four quadrants, and measuring the time the animal spends in the target quadrant where the platform used to be, and compare this to the time spent in the other three quadrants of the pool. If the animal has developed a spatial strategy, it will spend most of its time searching for the now missing platform in the target quadrant of the pool. A mouse that did not develop a spatial strategy will spend an equal amount of time in all four quadrants of the pool.

Spatial strategy is dependent on the hippocampus whereas non-spatial strategy is not. This was first shown in rats (Morris et al., 1982), but has since then been shown in numerous studies using mice (e.g. Angelo et al 2003). As in fear conditioning, the consolidation of spatial memory for the MWM requires protein synthesis (Meiri and Rosenblum, 1998) and CREB mediated transcription (Bourtchuladze et al., 1994; Gass et al., 1998; Kogan et al., 1997; Pittenger et al., 2002). Reconsolidation of spatial memories has been recently reported (Suzuki et al., 2004).

1.2.2 Systematic expression screens

Like the formation of memory, most biological processes rely on changes in gene expression. Comparison of gene expression in different samples provides a means to understanding the mechanisms underlying these biological processes. Such comparisons were originally done at the single gene level. Researchers were interested in a particular gene and would look at whether the expression of this gene would differ between samples. In the early nineties, systematic screens were developed that would allow the comparison of

the entire gene expression profile of tissues. Such screens have since generated gene expression data on a genomic scale and have identified new unknown genes as well as new roles for known genes.

Many types of systematic screens exist and rely on different molecular biology techniques. The screens differ in several aspects including sensitivity, reliability and costs. In this thesis, two different systematic screens were used and are described below: differential display (DD) and Affymetrix microarray (AM).

1.2.2.1 Differential display technology

Differential display is a simple and relatively inexpensive method that allows simultaneous visualisation and comparison of gene expression pattern between two or more samples isolated from cells/tissues under different conditions (Liang and Pardee, 1992). It relies on the combination of three frequently used molecular biology techniques: (i) reverse transcription of polyadenylated (poly A) RNA into complementary DNA (cDNA), (ii) polymerase chain reaction (PCR) of the cDNA and (iii) polyacrylamide gel electrophoresis (reviewed by Stein and Liang, 2002).

First, total RNA extracted from the tissues/cells of interest is reverse transcribed using reverse transcriptase and one of three anchor primers designed to anneal to the 3' poly-A tail of messenger RNA (mRNA). The resulting cDNAs are then used as templates for PCR amplification, using the same anchor primer as that used in the reverse transcription reaction in combination with an arbitrary primer (Appendix 1), usually 10-13 base pairs long. The PCR product is then run on a polyacrylamide gel resulting in an "expression fingerprint" where the multiple cDNA species derived from a given sample can be visualised. If different samples of interest are run side by side, then they can easily be compared, and

differences in gene expression can be detected (Fig. 1.6). After the DD is performed, bands with altered expression are selected, cut out of the gel and identified by sequencing. For each identified gene, the changes in expression then need to be confirmed using for example Northern blots, quantitative real-time PCRs, *in situ* hybridisations or RNAse protection assays. Indeed, false positives have been reported to make up a significant proportion (as high as 50-70%) of the differentially expressed bands (Reviewed by Debouck, 1995). The main reasons for this high rate of false positives is attributed to the presence of multiple cDNA fragments in one particular band, and competition by cDNA species of different abundance for PCR primers (Debouck, 1995).

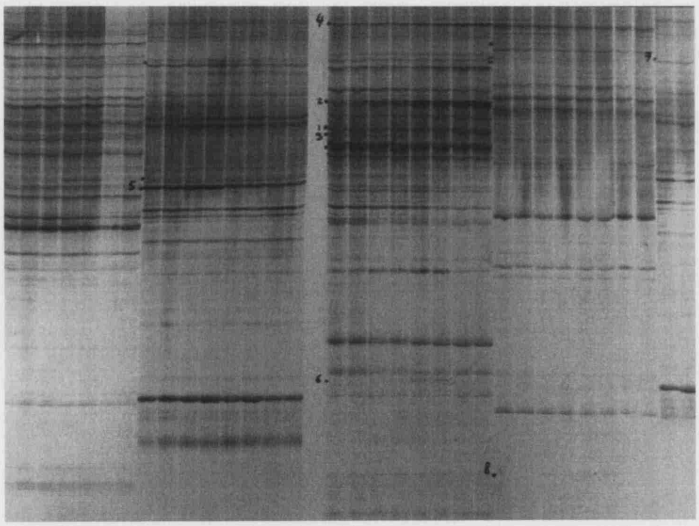

Fig. 1.6: Typical differential display gel.

1.2.2.2 Microarray technology and Affymetrix microarrays

Microarrays have been enthusiastically applied in many fields of biological research. As their name indicates, micorarrays rely on array technology: DNA is spotted on miniaturised arrays, and these arrays are screened with DNA/RNA derived from biological samples.

DNA/RNA species which are common between the array and the sample under study will hybridise together, can be detected and expression levels can be compared.

The first microarray technology to be widely used was the spotted cDNA microarray, which typically consists of probes of PCR-amplified cDNA fragments deposited in a matrix pattern of spots on a treated glass surface (Fig. 1.7 A)(DeRisi et al., 1996; Lockhart et al., 1996). cDNA is derived from two cell/tissue populations to be compared. The two cDNA populations are tagged with different fluorescent dyes, usually red and green. They are then pooled in equal amounts and hybridised to the same microarray (competitive hybridisation). The array is scanned for fluorescence and analysed. cDNAs that are expressed in both cell populations will fluoresce in both green and red. Those specific to one cell population will fluoresce in one colour only: green or red. By analysing and comparing the intensity of both green and red fluorescence for a particular cDNA, the abundance of that cDNA in one sample relative to the other sample can be determined (Fig 1.7A).

The microarrays that were used in this thesis are Affymetrix GeneChip microarrays (Affymetrix). The arrays are manufactured using photolithographic technology, and in contrast to cDNA microarrays, consist of *in situ*-synthesised oligonucleotide probes. Each gene target is probed by a number of distinct probes (11-20) collectively termed a probe set (Fig. 1.7B). Each probe set consists of Perfect Match (PM) and Mismatch (MM) probes. The PM probes are perfectly complementary to the target sequence. Each PM probe has a partner MM probe, which is identical except for a single base mismatch in its centre. This PM/MM design allows the detection, quantification and subtraction of signals caused by non-specific hybridisation. Biotinylated cRNA derived from a biological sample is hybridised onto the microarray; one sample per array. Therefore this is not a competitive hybridisation, as the expression of the genes is not compared on the same array (as with cDNA microarrays), but instead between two or more different arrays. The hybridised

microarrays are then labelled, scanned for fluorescence and analysed. To detect changes in gene expression, the amount of florescence for each gene is compared between the arrays (Fig.1.7B).

Affymetrix supply arrays for gene expression studies in a wide range of organisms including yeast, *Arabidopsis*, *Drosophila*, mouse, rat and human. The Murine Genome MG-U74v2 Set of Affymetrix microarrays contains probes interrogating approximately 36,000 full-length mouse genes and EST clusters. For the experiments described in this thesis, the mouse MG-U74Av2 chips were used, which contain probes for approximately 12 000 mouse genes. A more detailed description of the Affymetrix GeneChip array technology is available on the company's web site (www.affymetrix.com).

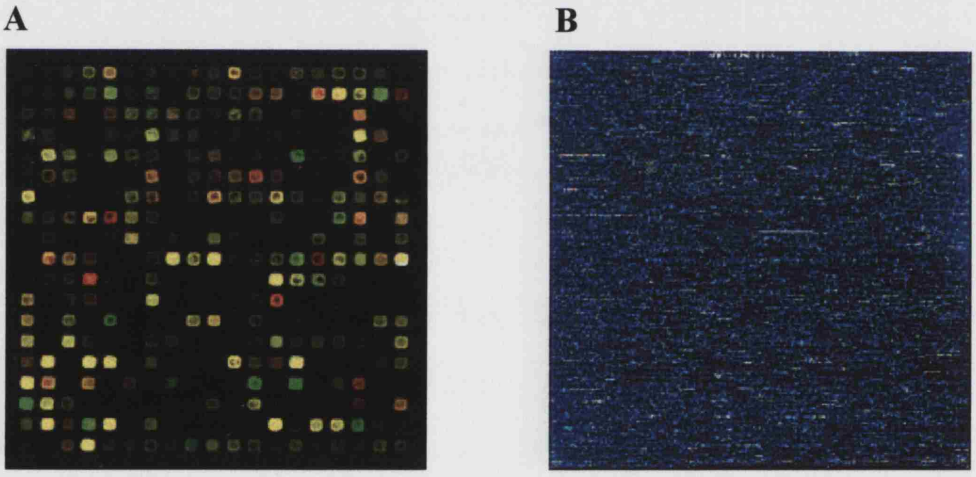

Figure 1.7 Two different types of microarrays (from mgm.duke.edu/genome/dna_micro/core/ protocols .htm). **(A)** Custom spotted cDNA microarray. Each spot represents a cDNA clone; **(B)** Affymetrix GeneChip. Each spot represents an oligonucleotide.

1.2.3 Real-time reverse transcription PCR (qPCR)

Reverse transcription polymerase chain reaction (RT-PCR) -based assays are the most common method for characterising or confirming gene expression patterns, and for comparing mRNA levels in different sample populations (Orlando et al., 1998; Suzuki et al., 2004). In the nineties, standardised, quantitative, fluorescence-based real-time RT-PCR (qPCR) was developed and introduced (Heid et al., 1996; Higuchi et al., 1993; Nazarenko et al., 1997; Woo et al., 1998). Nowadays, the technique can accurately detect variation in template levels of as little as 20% (Bustin, 2000).

The technique is based on the detection of a fluorescent reporter, the signal of which increases as the amount of PCR product accumulates. The fluorescent reporter can be (i) SYBR Green, a dye that binds double stranded DNA, or (ii) a sequence specific oligonucleotide probe (TaqMan Probe or Molecular Beacons). SYBR Green is an intercalating agent that emits fluorescent light upon irradiation when bound to double stranded DNA (dsDNA). Therefore, the more dsDNA is present in the reaction, the more fluorescence is emitted. TaqMan Probes and Molecular Beacons rely on fluorescence resonance energy transfer (FRET) for quantification. The probes contain a fluorescent dye and a quenching dye. When the fluorescent dye is irradiated in the vicinity of the quenching dye, the energy of the excited fluorescent dye is transferred to the nearby quenching dye resulting in non-fluorescence (process termed FRET). During PCR, the fluorescent and the quenching dye on the TaqMan probes or the Molecular Beacons become physically separate from each other (due to probe degradation in the case of TaqMan probes and probe linearization in the case of Molecular Beacons) and FRET no longer occurs and fluorescent light is emitted. Fluorescence increases in each cycle, proportional to the rate of DNA amplification. In this study, the SYBR Green was used as reporter.

When performing the qPCR, total RNA extracted from the tissues/cells is first reverse transcribed using reverse transcriptase and an anchor primer designed to anneal to the 3' poly-A tail of mRNA. The resulting cDNA is then used as a template for the qPCR amplification, in the presence of one of the fluorescent reporters. The amount of PCR product is monitored throughout the amplification. During the exponential phase of the PCR reaction, the amount of cDNA product present in each reaction is directly proportional to the amount of fluorescence emitted. The relative amount of cDNA can therefore be compared between samples and changes in gene expression detected (Fig. 1.8).

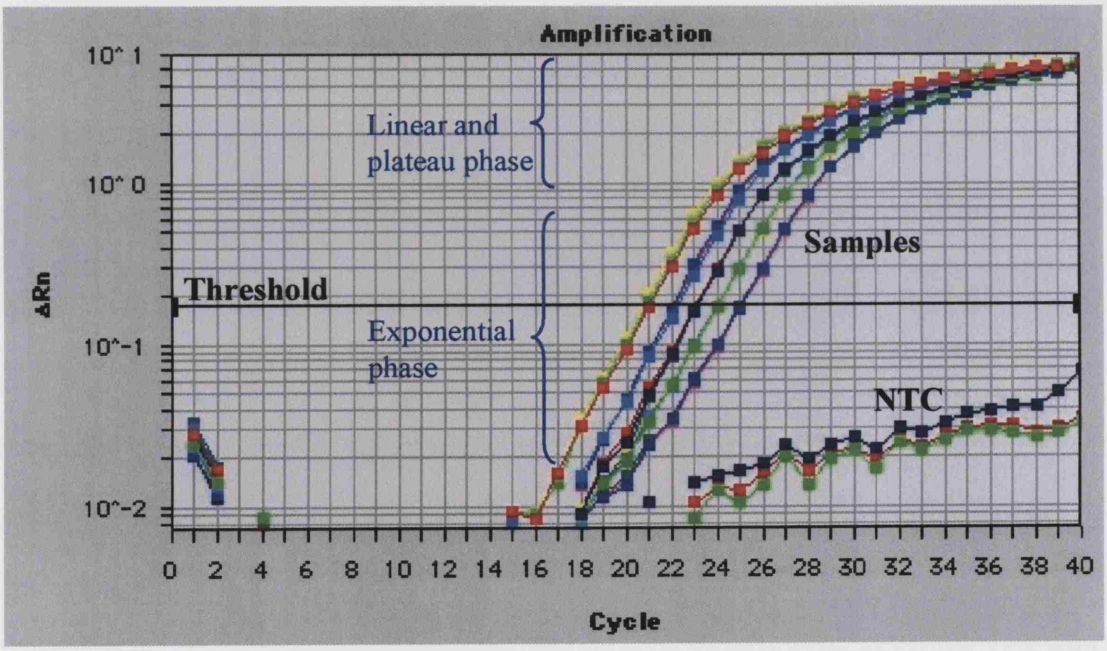

Figure 1.8 Typical quantitative real-time PCR amplification curve [Log(fluorescence) v cycle no].
X axis: cycle number of the quantitative PCR. *Y axis*: ΔRn (*Delta Rn*), reporter fluorescence, normalised to the fluorescence of the background and an inert reference fluorochrome. NTC: non-template control
When the PCR reagents are in excess and the template and product are at low concentrations, product renaturation does not compete with primer binding to the template. The amplification proceeds at a constant, exponential rate. At n cycles, exponential amplification slows to a linear phase, and then to a plateau phase (at which little/no product is made). To compare the template levels in different samples, one determines the threshold cycle (C_T) for a chosen value of ΔRn when all the reactions are proceeding exponentially. For the work presented in this thesis, a comparative C_T method was used, in which the amount of the target (determined from the C_T) is normalised to an endogenous reference (see material and methods section).

1.3 AIM OF THE PROJECT

At the start of this PhD project in spring 2001, studies on expression changes occurring during memory consolidation were sparse and only few of the genes regulated during memory consolidation had been identified. Furthermore, the idea of reconsolidation had only recently been reintroduced to the field of learning and memory (Nader et al., 2000; Przybyslawski and Sara, 1997) and little was understood about the phenomenon of reconsolidation. In particular, no study reporting expression changes during memory reconsolidation had been published.

The aims of this project were therefore:

1) To identify genes, whose expression is regulated during memory consolidation and/or reconsolidation
2) To investigate whether reconsolidation recapitulates consolidation at the molecular level. For this, the transcriptional events occurring during memory consolidation and reconsolidation were to be compared, using contextual fear conditioning as a behavioural task.
3) To investigate the function of at least one of the identified genes in L&M.

CHAPTER II:
MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 Housing

All subjects were housed in groups of 2-4 and maintained on a 12-h light-dark cycle with food and water *ad libitum*. All experiments were undertaken in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.1.2 Strains

2.1.2.1 Expression studies

Two- to five-month-old C57BL/6Jx129S2/SvHsd F1-hybrid male mice (Harlan, UK) were used for all expression studies, except for the study with the αCaMKII^{T286A} (T286A) mutants and NGFI-B null mutants. Homozygous T286A mutants (4 females and 2 males) and wild-type (WT) littermates (4 females and 2 males; 3-4 month-old) in the 129B6F2,3 hybrid background were obtained from intercrosses of heterozygous T286A mutants. Offspring were genotyped by PCR, as described in Giese et al. 1998. Homozygous NGFI-B null mice (Lee et al. 1995) (n = 11) and WT littermates (n = 8) in C56BL/6 background (2-5 month-old) were obtained from intercrosses of heterozygous NGFI-B null mice. Offspring were genotyped by PCR, using the primers NGFI-B_{WT}-FWD (5'-GTACACCGGA GAGTTTGACA CCT-3'), NGFI-B_{WT}-REV (5'-CATAGTACTCAGA GGGGCTGGAG-3') and PKG_{NEO} (5'-GAAGGAGCAAAGCTGCTATTGGCCGCTGCC-3'); the amplification conditions were: 93°C for 2 min, 35x[93°C for 30 s, 60°C for 45 s, 72°C for 45 s], 72°C for 10 min. All mice were re-genotyped after sacrifice.

2.1.2.2 Behavioural studies

Anisomycin study

For the anysomycin study, 8-12 week old C57BL/6Jx129S2/SvHsd F1-hybrid male mice were used.

Setup of the LI protocol

Initially, two- to three-month old C57BL/6J male mice (Charles Rivers, UK) were used for setting up the latent inhibition (LI) protocol and assessing the freezing. For the LI expression studies C57BL/6Jx129S2/SvHsd F1-hybrid male mice were used as described above.

NGFI-B functional study

For all studies, 2-5 month old NGFI-B mutants and WT littermates were used. The same mice were used for fear conditioning and Morris water maze. These mice were genotyped as described above.

For the fear conditioning studies, the mice were tested in three groups (*group 1*: 3 WT female, 4 WT male, 5 MUT female, 6 MUT male; *group 2*: 3 WT female, 5 WT male, 6 MUT female, 4 MUT male; *group 3*: 2 WT female, 5 WT male, 1 MUT female, 5 MUT male). *Group 1* and *3* were tested 24 hours after conditioning, whereas *group 2* was tested 28 days after conditioning.

For the Morris Water maze study, NGFI-B null mutant and WT littermate mice were studied in the MWM in three experimental groups (*group 1*: 3 WT female, 4 WT male, 4 MUT female, 4 MUT male; *group 2*: 3 WT female, 5 WT male, 6 MUT female, 4 MUT male; *group 3*: 1 WT female, 4 WT male, 1 MUT female, 3 MUT male). Conditions were kept constant throughout the groups.

2.2 BEHAVIOURAL AND PHARMACOLOGICAL PROCEDURES

2.2.1 Anisomycin administration

Anisomycin was administered systemically immediately after memory reactivation performed 24h after training. Anisomycin (Sigma, St. Louis, MO) was dissolved in acid saline (0.9% NaCl, pH = 2-3), the pH of the solution was subsequently adjusted to 7.0-7.4. The mice were injected intraperitoneally with either 150 mg or 225 mg anisomycin/kg body weight or an equivalent volume of 0.9% saline.

2.2.2 Contextual and cued fear conditioning

2.2.2.1 Behavioural equipment

Contextual and Cued conditioning were performed in a dim room. The conditioning chamber consisted of an ethanol-scented rectangular box, 27.5 cm wide (the front and back sides of the chamber), 12.5 cm deep (lateral sides of the chamber) and 14.0 cm high (Fig. 2.1A) (Campden Instruments). The lateral sides and ceiling of the chamber were made of aluminium, the front and back sides of Plexiglas. The front side was mounted on hinges and thus served as a door through which the animals could be placed into and removed from the chamber. The chamber had four white lights: one on each lateral side and two on the ceiling. The chamber had a metal grid floor connected to a constant voltage generator (521/C, Campden Instruments) via a shock scrambler (521/S, Campden Instruments) which was set to deliver a 0.75mA alternate current foot shock when switched on. A speaker, mounted in

the centre of the ceiling, delivered a 2.8 KHz tone at an intensity of 80 dB when switched on. For contextual fear memory testing, the mice were placed back into the conditioning chamber.

For cued fear memory testing, a hemi-circular lemon-scented box, 29.5 cm in diameter and 14.0 cm high was used (Fig 2.1B) (Campden Instruments). The sides of the chamber where made of Plexiglas, the hemi-circular side formed the back of the box, and the rectangular side the front of the box. Just as for the conditioning chamber, the rectangular front side was mounted on hinges and served as door through which the animals were placed into and removed from the box. The ceiling was made of aluminium, and the floor of a textured Plexiglas. The chamber had three red lights, two on the ceiling and one on the hemi-circular side of the box, and had the same speaker as that in the conditioning chamber mounted on the ceiling.

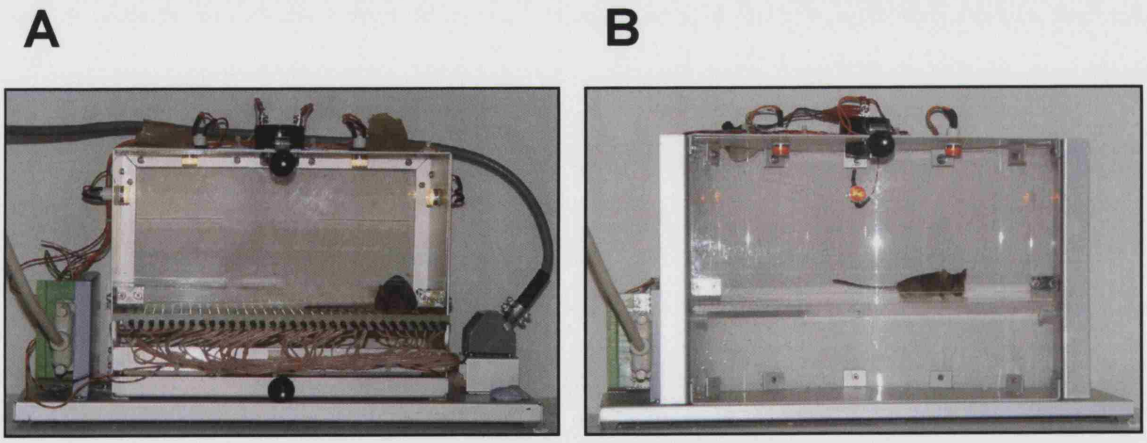

Figure 2.1 Fear conditioning chambers
(A) Training and context testing chamber **(B)** Cued testing chamber

Both chambers were placed in a sound-attenuating box (width: 60.5 cm, depth: 35.0 cm and height: 35.0 cm) fitted with a fan to mask background noises. The different behavioural protocols were computer controlled by the Animal Behavior Environment Test System software (ABET, Lafayette Instrument Company). All sessions were recorded via a video-camera connected to a VHS recorder.

2.2.2.2 Behavioural procedure

The mice were transported to the lobby adjacent to the behavioural room at least one hour before the onset of conditioning or testing. Each mouse was individually transported from the lobby to the behavioural room.

Background contextual fear conditioning (contextual fear conditioning with tone presentation) was used for all experiments. Background contextual conditioning seems to involve the hippocampus more strongly than conditioning without tone presentation (foreground conditioning) (Phillips and LeDoux, 1994). Furthermore, background, but not foreground conditioning induces the autophosphorylation of αCaMKII in the hippocampus (Atkins et al., 1998). Finally, background conditioning allows testing for both contextual and cued memory in the same individual.

Each mouse was placed into the conditioning chamber and after a 120 s introductory period, a tone (80 dB, 2.8 kHz) was presented for 30 s, which co-terminated with a 2 s foot shock (0.75 mA). After a further 30 s the mouse was returned to its home cage. The conditioning chamber was cleaned with water and wiped with ethanol between each mouse.

For contextual re-exposure/testing, the mice were returned to the conditioning chamber for 5 min, in the absence of tone or shock. For cued testing, the mice were placed in the hemi-

circular chamber for 6 min. For the first 3 min, no tone was played (pre-CS period), after which the tone (80 dB, 2.8 kHz) was played for another 3 min (CS period). Freezing was assessed every 5 s for 2 s; freezing was scored if no movements other than respiratory movements were detected.

2.2.2.3 Data analysis

For contextual conditioning, the freezing response was averaged over the 5 min testing period. For cued conditioning, the freezing responses during the pre-CS and the CS period were averaged separately. For statistical analysis one-way analysis of variance (ANOVA) and Student-Newman-Keuls post-hoc test were used.

2.2.3 The Morris water maze (MWM)

2.2.3.1 Behavioural equipment

The MWM consisted of a white circular pool, 150 cm in diameter and 50 cm deep. The pool was placed on a table (70 cm from the floor) to allow the experimenter to sit on the floor and remain concealed, and filled with water to approximately 20 cm below the rim of the pool. The water was made opaque through the addition of white non-toxic paint (ready mix paint, The Early Learning Centre, Swindon, UK), and kept at 26 ± 1°C throughout the experiment. A hidden platform, 0.5cm below the surface of the water and 10cm in diameter, was placed 30cm from the edge of the pool in the South West corner.

White curtains covering the walls of the behaviour room, at 55 cm away from each sides of the pool, surrounded the pool on its West, North and East sides. On the South side, curtains

were present but kept open to present the violet door of the room as a cue (Fig. 2.3). In addition to the door, four other cues were visible from the pool: (i) a set of two plastic footballs in a net, one red and one blue, close to the SW corner of the room; (ii) an orange plastic chair in the NW corner of the room; (iii) a two-dimensional black and white cardboard cue on the North side of the pool, close the NE corner; and (iv) a three-dimensional black and grey cardboard cue in the centre of the East facing wall. The room was lit by indirect light from four lamps, placed in each corner of the room and projecting light upwards.

The behaviour of the mice was monitored via a camera mounted on the ceiling, directly above the centre of the pool. The image was sent to a tracker (HVS2020 Tracker, HVS Image Ltd) connected to a PC running the HVS Water 2020 software (HVS Image Ltd.). This allowed the mice in the pool to be tracked and their swimming to be behaviour analysed.

2.2.3.2 Behavioural procedure

Prior to training, the mice were handled for 2 min each for 6 days, in order to reduce anxiety levels. Each mouse was individually placed on the experimenter's hand, and allowed to explore the hand, but not to climb up the arm.

Before the onset of each training session, the mice were acclimatised to the dim light condition of the MWM behaviour room for one hour. The home cages were placed under the pool in such way that the maze itself and all relevant cues were not visible. The cages remained in the behavioural room throughout each training session.

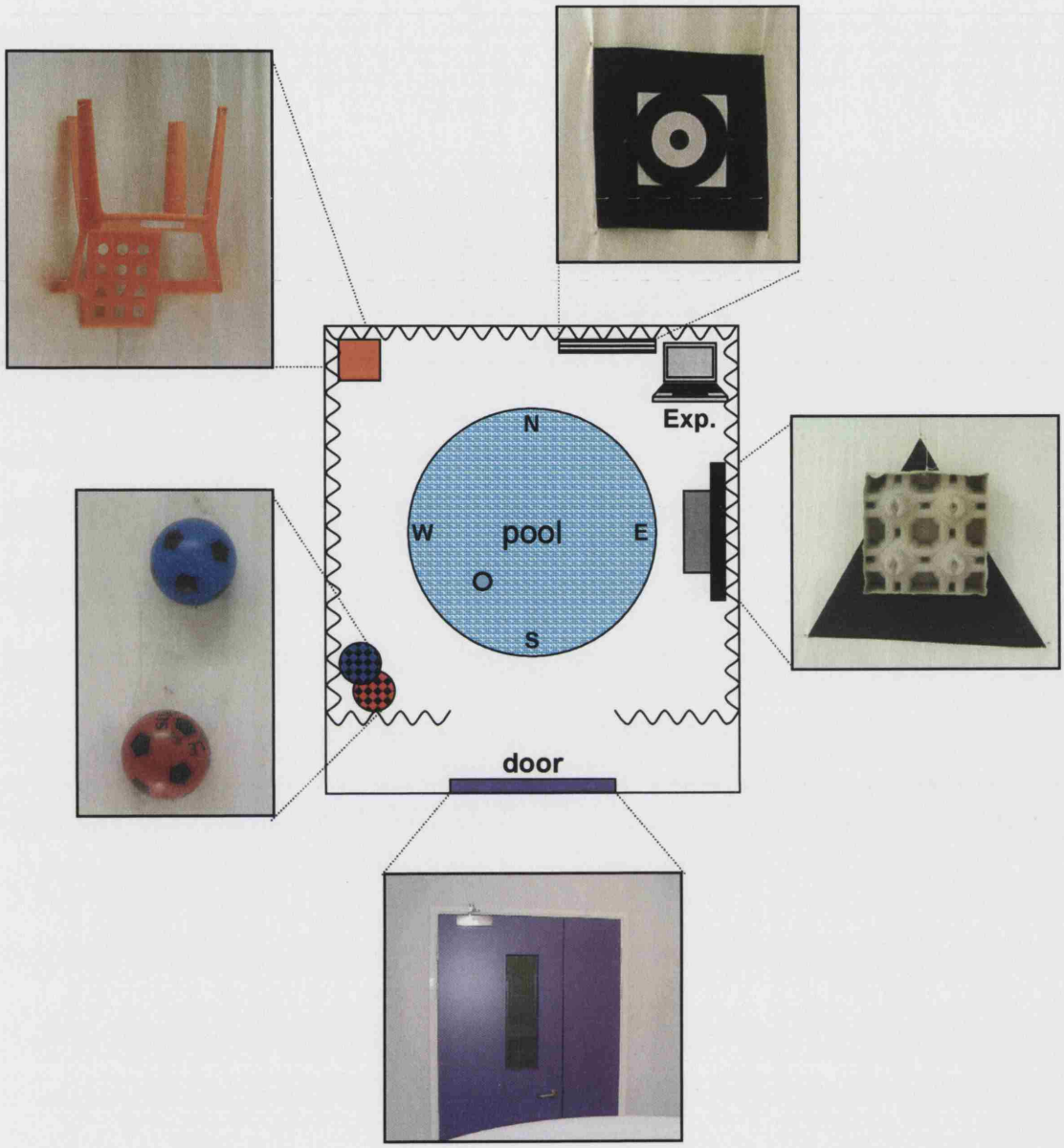

Figure 2.2 Morris water maze setup
Exp. = experimenter; ○ = platform; ∿∿ = curtains

Each mouse was tested individually. On the first day of training, the mice were first placed onto the hidden platform for 30s after which they where gently pushed off the platform and left to swim for another 30 s. The mice were then picked up, placed close to the hidden platform and guided to the platform from four different angles (North, East, South and West, respective to the pool). The first training trial started immediately after this habituation exercise.

The mice were trained for four trials per day for 9 days. Before the first trial of each day, the mice were placed onto the platform for 60 s. Then the mice were placed into the MWM, facing the pool wall, from four different starting positions (N, E, S and W) and allowed to search for the platform. The order of the four starting positions (e.g. E→S→N→W) was identical for each mouse for a given training day, but varied pseudo-randomly from day to day. A trial was ended and the escape latency scored when the animal had climbed onto the platform. The maximum trial length was 90 s and the inter-trial interval was 60 s, during which time the mouse was allowed to rest on the platform. If the animal had not found the platform within 90s it was removed from the water and placed back onto the platform, and a latency of 91s was scored. After the last trial the mouse was returned to the home cage.

On day 5 and day 9, a probe trial was performed after the training session to assess the search strategy of the mice. Each mouse was first place onto the platform for 60 s. The platform was then removed and the animal was placed into the MWM from the NE corner of the room and allowed to search for 60 s for the now missing platform, after which it was returned to its home cage.

2.2.3.3 Data analysis

Mice were excluded from the experiment if they:

1) never found the platform position in the first 3 days of training
2) showed excessive Floating (<5cm/s):
   a. more than 50% of time spent floating in 50% or more trials on at least two consecutive days (excluding day 1),
   b. more than 50% of time spent floating during the probe trial.
   c. more than 25% of time spent floating in 50% or more trails on at least two consecutive days from day 6 onwards (day 6: 1st day after 1st probe trial)
3) showed excessive thigmotaxis: spend more than 50% of time in the thigmotaxis zone in 75% or more trials on two consecutive days (excluding day1). The thigmotaxis zone was defined as the ring occupying the outer 10% of the pool (i.e. area within 7.5cm from the wall of the pool), which corresponded to 19% of the total pool area. The random level of thigmotaxis was therefore 19% of the time spent in the thigmotaxis zone.

For statistical analysis of the escape latencies, the average escape latency per mouse per block (i.e. training day) was first calculated. These mean escape latencies were then analysed by two-way ANOVA with repeated measures with genotype and block as factors. The Student-Newman-Keuls post-hoc test was used.

For statistical analysis of the probe trial data, one-way ANOVA and the Student-Newman-Keuls post-hoc test was used. Kruskal-Wallis one-way ANOVA on Ranks and the Student-Newman-Keuls post-hoc test were used when the data were not normally distributed.

2.3 MOLECULAR BIOLOGY

2.3.1 Hippocampal dissections

Mice were anaesthetised with CO_2, killed by cervical dislocation, and the brains removed and placed onto a sterile RNase-free dish. The hippocampi were quickly isolated under a low magnification microscope, placed into sterile eppendorf tubes that had been pre-chilled on dry ice, and stored at -80°C.

The hippocampus was extracted from the brain as follows:

1) The brain was placed on the dish, the neocortex facing upwards, and the olfactory bulbs pointing towards the experimenter, and the cerebellum pointing away from the experimenter.

2) A large incision along the midline was performed, cutting the corpus collasum.

3) Then, in the left hemisphere, the cortex together with the left side of the hippocampus was gently pushed towards the side, detaching the hippocampus from the underlying thalamic structures. The hippocampus was then rolled off the cortex, making sure that the most ventral part of the hippocampus was also isolated.

4) Procedure 3) was repeated for the right hemisphere.

2.3.2 Differential display screening

2.3.2.1 Hippocampal mRNA isolation

The frozen hippocampi were homogenised in TRIzol reagent (Invitrogen) and the total RNA isolated according to the manufacturer's instructions. The total RNA was cleared from any contaminating genomic DNA by lithium chloride precipitation. The quality of the RNA was

checked by gel electrophoresis, to assess the integrity of the 5s, 18s, 18s ribosomal RNA bands.

2.3.2.2 "Random priming" single-stranded cDNA synthesis

400ng aliquots of purified RNA were reverse transcribed, using 400 units of Superscript II reverse transcriptase (Invitrogen) in a 40μl reaction mixture containing first strand buffer (supplied by the manufacturer), 10mM dithiothreitol (DTT), 20mM dNTPs, 40μM anchor primers, 117 units of RNase inhibitor (Invitrogen). The anchor primers used in this study were $T_{12}VA$, $T_{12}VC$ and $T_{12}VG$, where V is a mixture of A, C and G (See Appendix I). The reaction was incubated at 42°C for 50 min. Following cDNA synthesis, the presence of genomic DNA contamination in the synthesised cDNA was checked using a PCR that distinguishes between genomic DNA and cDNA for the hypoxanthine phosphoribosyltransferase (HPRT) gene. The primers were: HPRT forward: 5'-GCTGGTGAAAAGGACCTCT-3' and HPRT-reverse: 5'-CACAGGACTAGAACACCTGC-3', and the amplification conditions were: 93°C for 2 min, 35x [93°C for 30 s, 58°C for 45 s, 72°C for 1 min], 72°C for 10 min.

2.3.2.3 Differential display polymerase chain reaction (PCR)

An aliquot (2μl) of the reverse transcription reaction was amplified using 1.5 units of Ampli Taq DNA polymerase (PE Biosystems) in a PCR mixture (20μl) containing PCR buffer (supplied by the manufacturer), 2μM dNTPs, 150 nmole of [α-^{35}S] dATP (1000 Ci/mmol; Amersham Pharmacia Ltd), 1μM of the same anchor primer as used in the reverse transcription reaction and 2μM of one of 28 arbitrary primers (See Appendix I). PCR was performed in a thermal cycler (Perkin Elmer PCR system 9700), and the amplification

conditions were: 94°C for 2 min; 5x [94°C for 15 s, 40°C for 2 min, 72°C for 20 s], 35x [94°C for 30 s, 42°C for 2 min, and 72°C for 20 s], 72°C for 5 min.

2.3.2.4 Polyacrylamide gel electrophoresis

3.5µl of each PCR product was denatured at 95°C for 2 min in 2µl loading buffer [98% v/v formamide, 10mM EDTA pH8.0, Xylene Cyanol, Bromophenol blue] and separated by electrophoresis on a 6% polyacrylamide sequencing gel. The gels were dried and visualised by autoradiography. Each PCR product was loaded twice to assess changes due to loading errors.

2.3.2.5 Extraction of DNA fragments from the sequencing gel

The differentially expressed cDNAs of interest were cut out from the dried gels and the corresponding gels re-exposed to confirm that the correct cDNA band had been extracted. The cut bands were eluted in 100µl Tris (pH 8.5) and boiled for 15 min. The eluted DNA was precipitated with 70% ethanol in the presence of 0.1µg/µl glycogen and 83mM NaOAC (pH = 5) and resuspended in 10µl H_2O.

2.3.2.6 Re-amplification of the extracted DNA fragments (Re-PCR)

An aliquot (2µl) of the cDNA solution was re-amplified using 0.6 unit of Ampli Taq DNA polymerase (PE Biosystems) in a PCR mixture (25µl) containing PCR buffer (supplied by the manufacturer), 0.1mM dNTPs and 1µM of the anchor and arbitrary primers specific to each eluted band. PCR was performed in a thermal cycler (Perkin Elmer PCR system 9700), and the amplification conditions were: 93°C for 2 min, 5x [93°C for 30 s, 40°C for 30 s, 72°C for 1min], 35x [93°C for 30 s, 42°C for 30s, and 72°C for 1min], 72°C for 5 min.

2.3.2.7 Cloning and identification of Re-PCR products

After confirming by electrophoresis on 1.2% agarose gel that the Re-PCR product showed a single or a dominant band of the predicted size, the re-amplified PCR products were cloned into the pCR 2.1 TOPO vector (Invitrogen) according to the manufacturer's instructions, and used to transform competent XL10 cells. The transformed bacteria were selected by an overnight incubation (37°C) on LB agar containing 100μg/ml ampicillin. Four to ten individual colonies from each transformation were sub-cultured overnight in LB-ampicillin broth at 37°C and the plasmid DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. An aliquot (1μl) of the miniprep DNA was digested with the restriction enzyme EcoRI and the size of the insert in each clone determined by electrophoresis on a 1.2% agarose gel. Plasmids containing an insert of the predicted size were sequenced by the WIBR sequencing service using the plasmid DNA for priming (T7 and M13-reverse primers). The identity, homology or novelty of the clones was determined by comparing the obtained sequence with the NCBI (National Center for Biotechnology Information) databases.

2.3.3 Affymetrix microarray screening

All the procedures were performed according to the Affymetrix GeneChip guidelines (see www.affymetrix.com).

2.3.3.1 Hippocampal mRNA isolation

The frozen hippocampi were homogenised in TRIzol® reagent (Invitrogen) and the total RNA isolated according to the manufacturer's instructions, and purified through RNeasy

mini-columns (Qiagen). The quality of the RNA was checked using the Agilent 2100 Bioanalyser. Ratios of rRNA [28S/18S] above 1.8 were considered acceptable.

2.3.3.2 Double stranded cDNA (ds-cDNA) synthesis

RNA (22 µg) from each sample was reverse transcribed using 600 units of Superscript II reverse transcriptase (Invitrogen) in 20µl reaction mixture containing first strand buffer (supplied by the manufacturer), 10mM DTT, 500µM dNTPs and 100pmol T7-(dT)24 anchor primer (synthesised by Invitrogen). The reaction was incubated at 42°C for 1 hour. Then, second strand synthesis was performed using 40 units of *E. coli* DNA polymerase I (Invitrogen) in a 150µl reaction containing second strand buffer (supplied by the manufacturer), 200µM dNTPs, 10 units of *E. coli* DNA ligase (Invitrogen) and 2 units of *E coli* RNase H (Invitrogen). The reaction was incubated at 16°C for 2 hours in a cooling water bath. The ds-cDNA was purified using phenol: chloroform: isoamyl alcohol (25:24:1) extraction, precipitated using ethanol, and resuspended in 12 µl ddH_2O. The ds-cDNA samples were checked for genomic DNA contamination using a PCR that distinguishes between genomic DNA and cDNA for the HPRT gene, as described in section 2.1.2.

2.3.3.3 Biotin-labelled cRNA synthesis and cRNA fragmentation

Biotin-labelled cRNA was synthesised from ds-cDNA (5µl) using the Bioarray High Yield RNA transcript Labelling kit (ENZO Life Sciences) according to manufacturer's instructions. The resulting cRNA was purified through RNAeasy mini-columns (Qiagen). The cRNA was quantified and its quality checked using the Agilent 2100 Bioanalyser. 20µg of cRNA was fragmented for 35 min at 94°C in 40µl fragmentation buffer (Affymetrix).

2.3.3.4 Hybridisation and scanning of Affymetrix chips

These procedures were carried out at the Affymetrix Facility of the Institute of Child Health (London) under the kind technical supervision of their staff. 10μg of fragmented cRNA was hybridised at 45°C to each Affymetrix GeneChip for 16 hours in an Affymetrix GeneChip Hybridisation oven using 200μl hybridisation buffer (supplied by manufacturer) containing eukaryotic hybridisation controls [bioB (91.5pM), bioC (5pM), bioC (25pM) and bioD (100pM)], 20μg Herring Sperm DNA, and 0.1mg Acetylated BSA. The chips were then stained and washed in an Affymetrix GeneChip Fluidics Station as follows: the chips were washed, stained for 10 min in Streptavidin-Phycoerythrin (SAPE) solution [MES Stain Buffer, 2mg/ml acetylated BSA, 10μg/ml Streptavidin-phycoerythrin], washed, stained in antibody solution [MES stain buffer, 2mg/ml acetylated BSA, 0.1mg/ml normal goat IgG, 3μg/ml biotinylated antibody], stained once more in SAPE, then washed again (for more detail, see manufacturer's instructions). After the last wash, the arrays were scanned using an Affymetrix GeneChip Scanner.

2.3.3.5 Data analysis

The affymetrix data was analysed using two different softwares, dChip (Li and Wong, Harvard University, USA) and GeneSpring (Silicon Genetics). Genes which showed significant changes using both softwares were chosen for further studies.

For the D-chip analysis, the arrays were first normalised to have comparable brightness (since scanned images may have different overall brightness), using the array with median overall intensity as the baseline array against which the other arrays were normalised. The level of expression of the different genes was then measured using two methods: the PM-MM Average Difference (AD) method and the PM Model Based Expression Index (MBEI),

as they show different sensitivities for detecting changes in expression depending on the level of transcript present in the samples.

For the GenesSpring analysis, the arrays were first normalised to have comparable brightness (per Chip normalisation), using control genes (housekeeping genes and reference genes form other organisms that should not hybridise) present on the array. Then, the level of expression of each gene was calculated by subtracting the overall average gene intensity (within a single experiment) from the raw intensity data for each gene, and dividing that result by the SD of all of the measured intensities (per Gene normalisation).

2.3.4 *In situ* hybridisation

2.3.4.1 Tissue preparation

Immediately after dissection the brains were frozen in isopentane cooled to between -20°C and -30°C, then transferred to dry ice prior to storage at -80C. 15 µm coronal brain sections were cut on a cryostat and thaw-mounted onto Superfrost slides (Invitrogen). The sections were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 5 min on ice, rinsed with PBS for 1 min, dehydrated in 70% ethanol for 5 min, and stored at 4°C in 95% ethanol.

2.3.4.2 Probe labelling

A 45-mer antisense DNA oligonucleotide complementary to the mRNA of interest was synthesised by Invitrogen UK. 10ng of oligonucleotide was end-labelled with 500nM [α-^{35}S] dATP (1000 Ci/mmol; Amersham Pharmacia Ltd) using 22.5 units of terminal deoxynucleotidyl transferase (Promega) in 12.5 µl transferase buffer (provided with

enzyme) for 15 min at 37°C. The reaction was terminated by the addition of 40µl TE, and the labelled probes were purified through columns made from Sephadex G25 (Sigma). 1µl of 1M DTT was added to the purified labelled probes.

2.3.4.3 Hybridisation

Air dried slides were hybridised at 42°C for 16 h in a humidified chamber with 50 µl hybridisation buffer [50% (v/v) formamide, 4x SSC pH 7.0, 25 mM sodium phosphate pH 7.0, 1 mM sodium pyrophosphate, 20 mM DTT, 2x Denhardt's solution, 200 µg/ml heat-denatured salmon sperm DNA, 10% (w/v) dextran sulphate, and 100,000- 300,000 cpm ^{35}S-labelled probe] per slide. Sections were washed twice in 1x SSC at 55°C for 30 min, transferred through 0.1x SSC, 70% ethanol and 95% ethanol, and air-dried. To control for non-specific hybridisation, adjacent sections were incubated with radiolabelled oligonucleotide in the presence of an excess concentration (100x) of unlabelled oligonucleotide probe.

2.3.4.4 Data analysis

The sections were exposed to a ^{35}S-sensitive film for autoradiography together with ^{14}C microscale standards (Amersham Bioscience, Bucks, United Kingdom) for 2 weeks at room temperature. The autoradiograph of every brain section was imaged with a monochrome camera. The resultant images were calibrated (nCi/g) with reference to the ^{14}C standards (nCi/g tissue equivalent (TE)), and the intensity of the signal quantified/analyzed using the MCID M5+ image analysis system (Imaging Research, St Catherines, Ontario, Canada).

For the NGFI-B expression study, the following 45-mer oligonucleotide was used: 5'-ATTGGTAGGGGAGGCATCTGGAGGCTGCTTGGGTTTTGAAGGTAG-3'. The

intensity of the signal was measured as the number of counts (1 count = 1 pixel) above the threshold density (29.63 nCi/g TE) at which the signal was detected in the control samples. The size of the CA1 areas analysed between naïve and trained mice were not significantly different (one-way ANOVA, $F_{1,46} = 0.12$, $p = 0.73$) (data not shown).

2.3.5 Quantitative real-time polymerase chain reaction (qPCR)

2.3.5.1 RNA isolation and cDNA synthesis

Hippocampi were fresh frozen on dry ice and stored at –80°C. Total RNA was extracted using Trizol (Invitrogen) and purified through RNeasy mini-columns (Qiagen). RNA (4 μg) from each sample was reverse transcribed using 200 units of superscript II reverse transcriptase (Invitrogen) in 20μl reaction mixture containing first strand buffer (supplied by the manufacturer), 10mM dithiothreitol, 500μM dNTPs and 0.5μg of oligo $(dT)_{12\text{-}18}$ primer (Invitrogen). The obtained cDNA was diluted 1/10 and stored at –20°C. The cDNA samples were checked for genomic DNA contamination using a PCR that distinguishes between genomic DNA and cDNA for the HPRT gene (as described in section 2.1.2).

2.3.5.2 qPCR

Real-time PCR primers were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and are shown in Appendix 2. qPCR was performed on the ABI7000 PCR system (Applied Biosystems) using SYBR Green as a fluorescent marker. For each sample, triplicates of 25 μl reactions were prepared containing SYBR Green PCR master mix (Applied Biosystems) along with 1 μl of cDNA and the appropriate final primer concentration (Appendix 3), which was determined empirically. The reaction was performed

in MicroAmp Optical 96-well reaction plates (Applied Biosystems) capped with an ABI PRISM Optical Adhesive Cover (Applied Biosystems). The PCR product levels were continuously measured by ABI7000 during 40 cycles. The cycle conditions were: 50°C for 2 min followed by 40 x [95°C for 15 s, 60°C for 1 min]. The primer concentrations were optimised so that the amplification efficiencies of both the template and the endogenous HPRT/GAPDH were identical throughout a wide range of mRNA dilutions. The specificity of the reaction was confirmed by sequencing the qPCR product. For this, the qPCR product was purified using a QIAquick gel extraction kit, according to manufacturer's instruction, and resuspended in H_2O. After each reaction, the product was checked for the absence of primer dimmers by performing a dissociation curve.

2.3.5.3 Data analysis

For each sample, the mean threshold cycle (C_T) for triplicate reactions was determined. The comparative C_T method was used in which the target mRNA amount was normalised to the endogenous HPRT or GAPDH mRNA amount, and calibrated to the mRNA amount in the naïve group. For statistical analysis one-way ANOVA or one-way ANOVA on ranks (when variance not equal), and Student-Newman-Keuls or Dunn's post-hoc tests were used. For the analysis of the T286A data, a two-way ANOVA was performed, with genotype and training as factors, and a Student-Newman-Keuls post-hoc test was used.

2.3.6 Genotyping of T286A point mutants and NGFI-B null mutants

Genotyping was carried out by PCR, with DNA obtained from tail biopsies on the day of weaning (P21). 5mm of mouse-tail was incubated in lysis buffer (100mM Tris-HCl (pH

8.3), 5mM EDTA, 0.2% SDS, 200mM NaCl, 0.1mg/ml proteinase K) overnight at 55°C. The digests were vortexed, centrifuged (10 min at 13,000 rpm), and the DNA extracted from the supernatant by an isopropanol precipitation.

The primers for T286A mutant mice:

T286A forward: 5'-CTGTACCAGCAGATCAAAGC-3'

T286A reverse: 5'-ATCACTAGCACCATGTGGTC-3'

The primers for NGFI-B null mutant mice:

NGFI-B_{WT}-Forward: 5'-GTACACCGGAGAGTTTGACACCT-3',

NGFI-B_{WT}-Reverse: 5'-CATAGTACTCAGAGGGGCTGGAG-3',

PKG_{NEO}: 5'-GAAGGAGCAAAGCTGCTATTGGCCGCTGCC-3'

The PCR reaction was performed using 1.5 units of Taq DNA-polymerase (Invitrogen) in a final volume of 25µl containing PCR buffer (supplied by the manufacturer), 100µM dNTPs (Perkin Elmer), 2mM $MgCl_2$ and 5pmol of each primer. The amplification conditions for the T286A PCR were: 93°C for 2 min, 35x [93°C for 30 s, 58°C for 30 s, 72°C for 30 s], 72°C for 10 min. The amplification conditions for the NGFI-B PCR were: 93°C for 2 min, 35x[93°C for 30 s, 60°C for 45 s, 72°C for 45 s], 72°C for 10 min. All mice used for behavioural experiments were re-genotyped once they had been sacrificed.

The sizes of the product for the T286A PCR are ~200 bp for WT band and ~250 bp for T286A mutant band. The sizes of the product for the NGFI-B PCR are 220 bp for the WT and 540 bp for the NGFI-B mutant band (Figure 2.3).

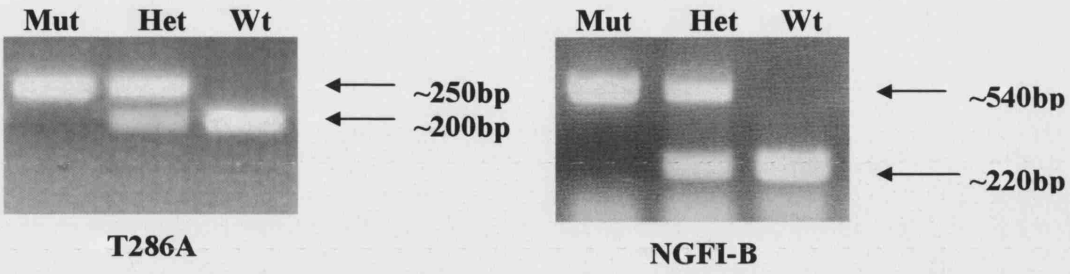

Figure 2.3 Typical PCR result for genotyping. Mut = mutant; Het = heterozygote; Wt = wild type

CHAPTER III:
RESULTS

3.1 EXPRESSION STUDY

To understand the molecular mechanisms underlying memory consolidation and reconsolidation, a systematic approach using contextual fear conditioning in combination with either PCR differential display or Affymetrix microarrays was used. The goal was to identify genes, whose expression is altered in the mouse hippocampus during hippocampus-dependent memory consolidation and/or reconsolidation. As *de novo* transcription and protein synthesis in the hippocampus are critical for consolidation and reconsolidation of contextual fear memory (Debiec et al., 2002; Dudai, 2004; Kida et al., 2002; Silva and Giese, 1994), genes that are engaged during these processes were likely to be detected in this way.

3.1.1 Preliminary control experiments

3.1.1.1 The conditioning protocol induces substantial contextual freezing

The contextual conditioning protocol used in the present study induced substantial freezing 24 hours after training (51.8 ± 5.6%), whereas the context without conditioning did not evoke freezing (0.5 ± 0.4%); one-way ANOVA showed a significant difference between the two groups (Fig. 3.1; $F_{1,14} = 48.6$, $p < 0.001$). Therefore, mice trained with this conditioning protocol develop a contextual fear memory and can be used to study expression changes occurring in the hippocampus during contextual memory consolidation. Furthermore, as the mice exposed to the context without conditioning did not develop a contextual fear memory, these mice can be used as a control group for gene expression induced by the environment alone.

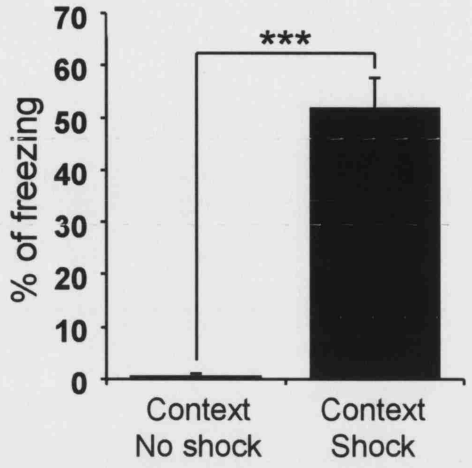

Figure 3.1: Contextual training induced freezing to context 24 h after training.
Two exposures to the context without conditioning did not evoke a freezing response, whereas exposure to the context after conditioning resulted in freezing. The freezing score during a second exposure, 24 h after the first exposure, is shown. ($n_{context\ no\ shock} = 6$, $n_{context\ shock} = 10$).

3.1.1.2 A five-minute re-exposure to the conditioning context triggers memory reconsolidation, but not extinction

To study expression changes occurring in the hippocampus during memory reconsolidation, mice were re-exposed for 5 min to the training chamber 24 hours after conditioning. To confirm that this re-exposure protocol induces protein synthesis-dependent reconsolidation, anisomycin was administered systematically immediately after re-exposure. Contextual freezing was assessed 24 hours later (Fig. 3.2A). Anisomycin significantly reduced contextual freezing ($ani_{(150mg/kg)}$ group = 25.7 ± 5.9% ; $ani_{(225mg/kg)}$ group = 21.3 ± 3.8%) as compared with the saline group (43.9 ± 5.6%; $p < 0.05$ for both) (Fig. 3.2B; one-way ANOVA, $F_{2, 26} = 5.1$, $p < 0.05$). Thus, the five-minute re-exposure protocol induces memory reconsolidation. Furthermore, freezing of the saline group on test 2 was not significantly

different from that on test 1 (Fig. 3.2C; one-way ANOVA, $F_{1, 22} = 1.4$, $p = 0.20$), indicating that our protocol did not induce extinction of contextual memory. The 5 min re-exposure protocol was therefore suitable for studying expression changes occurring during contextual memory reconsolidation.

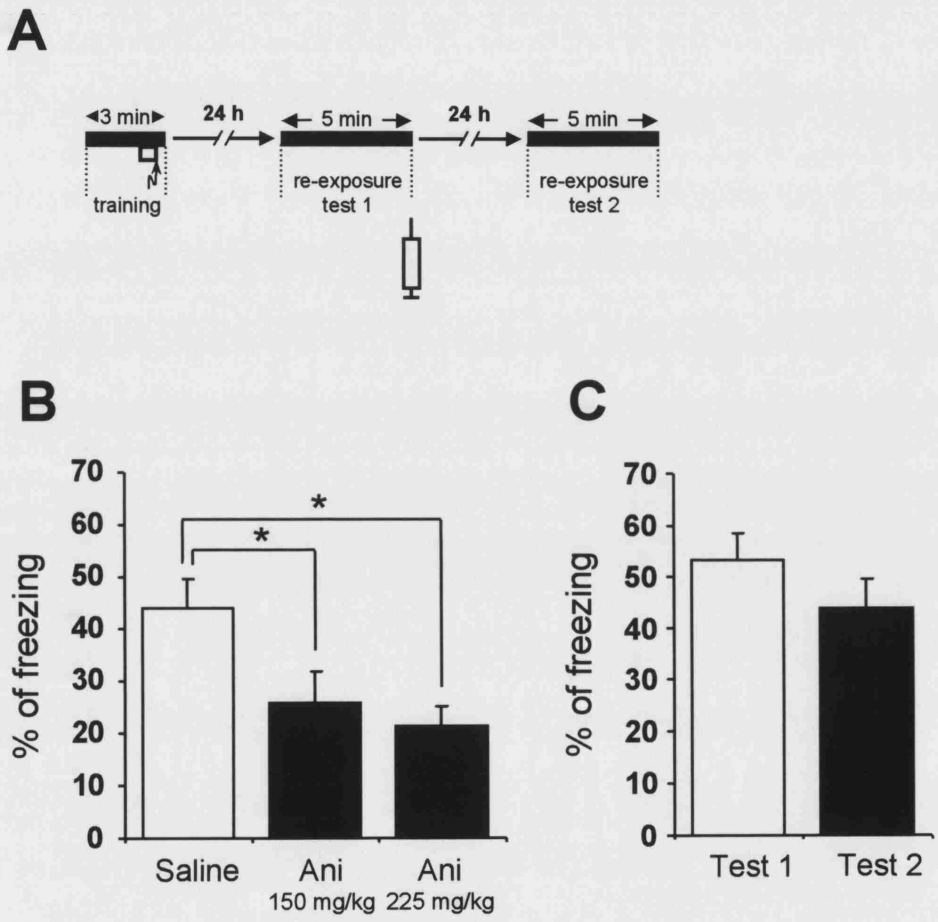

Figure 3.2: A five minute re-exposure to the training context triggers memory reconsolidation, but not extinction. Means ± SEM, * $p < 0.05$.

(A) Experimental procedure for anisomycin administration. The white box represents a 30 s tone, the arrow symbolizes the foot shock, the syringe indicates the intraperitoneal injection of anisomycin.

(B) Systemic anisomycin administration immediately after a 5 min re-exposure significantly reduced contextual freezing assessed 24 h after the re-exposure ($n_{saline} = 12$, $n_{ani(150mg.kg)} = 9$, $n_{ani\ (250mg/kg)} = 8$).

(C) Re-exposure to the context does not induce contextual memory extinction in the saline group.Two exposures to the context without conditioning did not evoke a freezing response, whereas exposure to the context after conditioning resulted in freezing. The freezing score during a second exposure, 24 h after the first exposure, is shown. ($n_{context\ no\ shock} = 6$, $n_{context\ shock} = 10$).

3.1.2 PCR differential display screening

3.1.2.1 Experimental groups

Blocking protein synthesis 3 hours after contextual fear conditioning inhibits LTM formation (Bourtchouladze et al., 1998). Hence, at this time point, mRNAs encoding proteins essential for LTM formation must be present in the hippocampus, and should be detectable. The three-hour post-training or post-re-exposure point was therefore chosen as the time point at which mRNA levels between conditioned and unconditioned animals were to be compared. Four different experimental conditions were used (each n=3) (Fig. 3.3A): *(i)* Box+3_{DD} group, exposed to the training context for 3 min in the absence of foot shock and tone, returned to their home cage and killed 3 h after exposure; *(ii)* T+3_{DD} group, early memory consolidation group, killed 3 h after training; *(iii)* T+27_{DD} group, late memory consolidation group, killed 27 h after training; *(iv)* R+3_{DD}, memory re-activation group, re-exposed to the training context 24 h after training and killed 3 h after re-exposure (i.e. 27 hours after conditioning). The T+27_{DD} group not only served as a control for the reconsolidation group, but also allowed determination of changes in gene expression occurring later during consolidation. Indeed, some studies have suggested that two or even more waves of protein synthesis exist during the consolidation period (e.g. Bourtchouladze et al., 1998).

Consistent with our previous experiments, the R+3_{DD} group froze substantially to the context 24 hours after training (Fig. 3.3B; 48.3% ± 7.3%), indicating that a contextual memory had formed, and that this memory was reactivated. This group could therefore be used to look at expression changes occurring in the hippocampus during contextual memory reconsolidation.

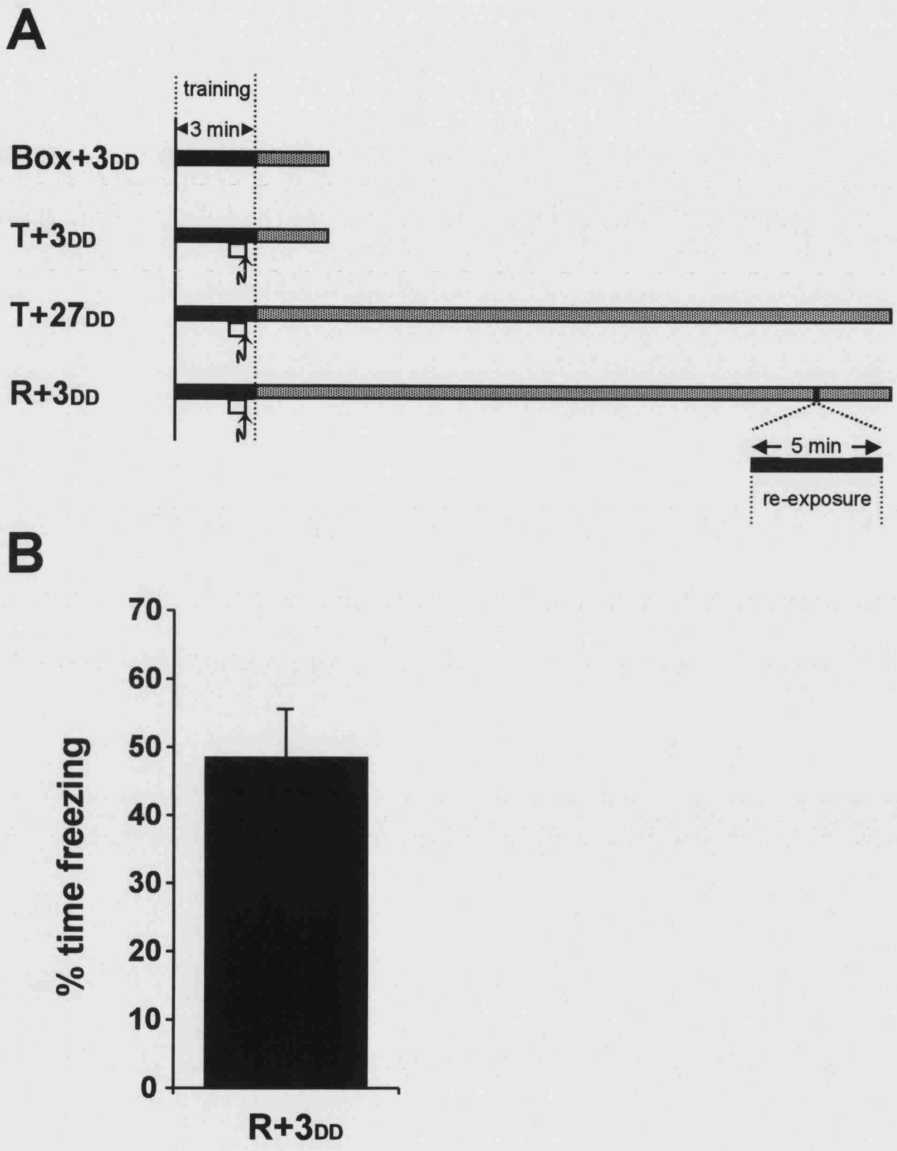

Figure 3.3 : Differential Display Experimental groups and contextual freezing scores.
(A) Experimental design to investigate changes in hippocampal mRNA expression induced by contextual conditioning. Black boxes indicate exposure to the training context, white boxes represent a 30 s tone, the arrow symbolizes the foot shock and the filled boxes show the time until the animals were sacrificed. The following groups were studied: Box+3_{DD}: exposed to training context and killed 3 h after exposure; T+3_{DD}: trained and killed 3 h after training; T+27_{DD}: trained and killed 27 h after training; R+3_{DD}: trained, re-exposed 24 h after training and killed 3 h after re-exposure.
(B) The R+3_{DD} group froze significantly to the context 24 hours after conditioning

3.1.2.2 Expression profiles observed with the PCR differential display

PCR differential display is a simple and relatively inexpensive method that allows simultaneous comparison of several RNA samples isolated from tissues under different conditions. The four groups detailed above were compared. For each group, the total RNA from the hippocampi of three animals was pooled, to reduce the chance of detecting false positive signals. The patterns of cDNA banding was compared on sequencing gels using 78 combinations of primer pairs (AR1-28 x TVA, TVC or TVG; see Appendix I for more detail) (Fig. 3.4). 138 cDNA bands showed clear changes in their expression profile, and are listed in Table 3.1.

Expression changes triggered by contextual conditioning

First, expression changes occurring during contextual memory consolidation were considered. This was determined by visual inspection. 134 cDNA bands showed noticeable changes in their intensities in the $T+3_{DD}$ and/or $T+27_{DD}$ group compared to the $Box+3_{DD}$ control group (Table 3.1). Interestingly, the number of down-regulated genes was almost as high as the number of up-regulated/newly-expressed genes, with 46% as compared to 54%. However, one has to be careful when considering these percentages, as the DNA sequence analysis was not performed for all 138 cDNA bands (see below) and thus one cannot be assert that these 138 cDNA bands correspond to 138 different transcripts. Indeed, some transcripts were detected more than once (see below). Therefore, no further percentages will be given here.

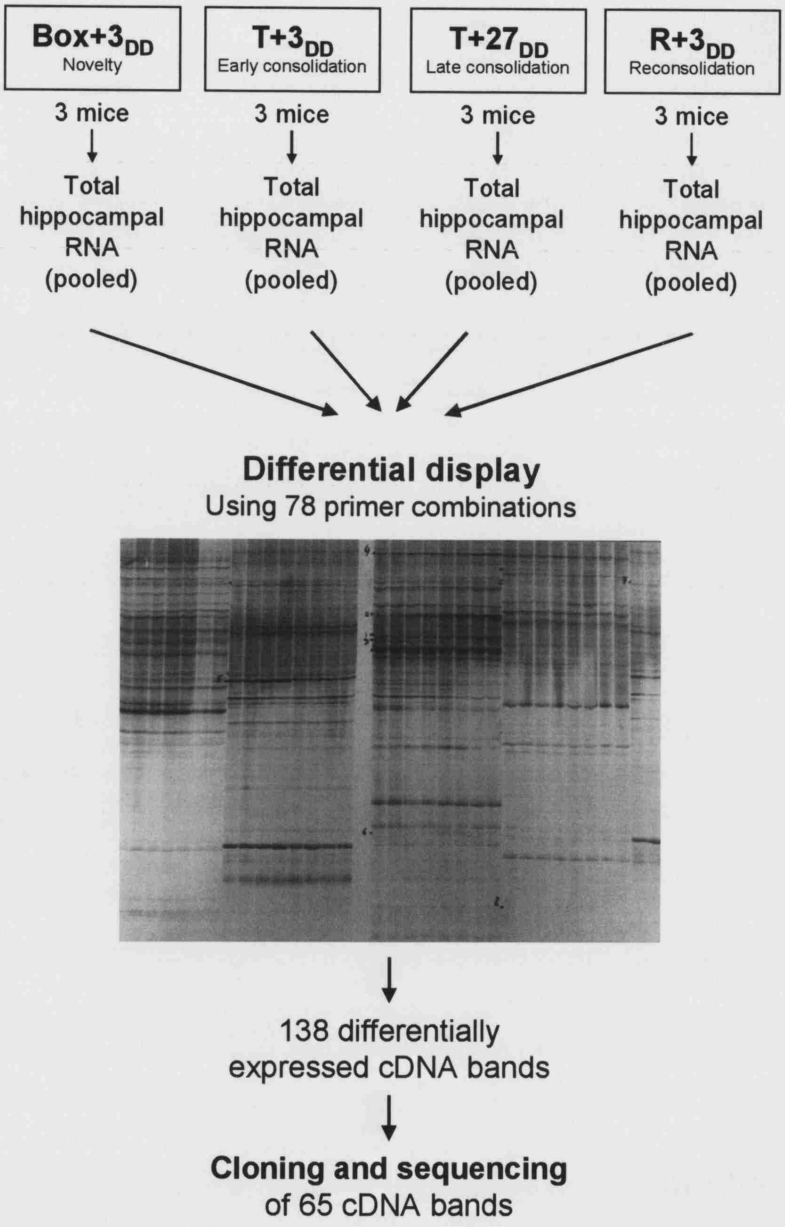

Figure 3.4 : Experimental summary for the Differential Display
Total hippocampal RNA for each group was pooled and cDNA synthesised. The cDNA was amplified using random decamer primers, and the PCR products run next to each other on a polyacrylamide sequencing gel. Each PCR product was loaded in duplicate. 138 differentially expressed cDNA bands were identified. 65 cDNA bands were extracted from the gel, cloned and sequenced.

#	Clone	B+3	T+3	T+27	R+3	#	Clone	B+3	T+3	T+27	R+3	#	Clone	B+3	T+3	T+27	R+3
1	A1	-	+	-/+	-	**47**	I7	++	+	+	+	**93**	M9	-	+	+	-
2	A2	+	-	-/+	-/+	**48**	I8	+	-	-	-	**94**	M10	+	-	-	-
3	A3	-	+	+	-	**49**	I9	+	++	++	+	**95**	M11	+	+	+	++
4	A4	-	+	+	-	**50**	I10	-	+	+	-	**96**	N1	-	+	-	-
5	A5	++	+	++	+	**51**	I11	-	+	-	-	**97**	N2	+	-	+	-/+
6	A6	+	-	+	+	**52**	I12	-	+	+/-	-	**98**	N3	+	++	+	+
7	A7	+	-	-	-	**53**	J1	++	+	+	+	**99**	N4	+	-	+	-/+
8	A8	+	-	-	-	**54**	J2	++	+	-	++	**100**	N5	+	++	++	++
9	B1	-	+	-/+	-	**55**	J3	++	+	-	++	**101**	N6	+	-	+	-/+
10	B2	+	-	-	-	**56**	J4	+	++	++	++	**102**	N7	+	++	++	++
11	B3	-	+	-	-	**57**	J5	++	+	+	+	**103**	N8	+	-	+	+
12	B4	-	+	-	-	**58**	J6	++	++	+	+	**104**	N9	+	-	+	+/-
13	B5	+	+	-	-	**59**	J7	++	+	-	++	**105**	N10	+	++	+	+
14	B6	+	-	-	-	**60**	J8	+	-	-	-	**106**	N11	+	++	+	+/++
15	C1	+	-	-	-	**61**	J9	+	-	-	-	**107**	N12	-	+	+	-
16	C2	-	+	+	-	**62**	J10	+	++	++	++	**108**	N13	+	++	++	+
17	C3	+	-	-	-	**63**	J11	+	++	++	++	**109**	N14	-	+	-	-
18	C4	-	+	+	-	**64**	J12	-	+	+	-	**110**	O1	+	+	+	++
19	D1	-	+	+	-	**65**	J13	-	+	+	-	**111**	O2	+	++	+++	+++
20	D2	+	+	++	++	**66**	J14	-	+	-	-	**112**	O3	+++	++	+	+
21	D3	++	+	+	+	**67**	J15	+	-	+	-	**113**	O4	+	++	++	+++
22	D4	++	+	+	+	**68**	J16	++	++	+	+	**114**	O5	+	++	+	+
23	D5	++	+	+	+	**69**	J17	+	-	-	-	**115**	O6	-	-	+	-
24	D6	++	+	+	+	**70**	J18	+	++	++	+++	**116**	O7	-	+	-	-
25	D7	+	++	++	++	**71**	J19	-	+	-/+	-	**117**	O8	-	+	+	-
26	D8	++	+	+	+	**72**	J20	++	+	+	+	**118**	O9	-	+	+	-
27	D9	++	+	+	+	**73**	J21	+	-	-	-	**119**	O10	+	+	-	-
28	D10	+	-	-	-	**74**	L1	+	++	+	+	**120**	P1	++	++	+	+
29	D11	+	-	-	-	**75**	L2	-	+	-	-	**121**	P2	++	++	+	+
30	G1	+	+	++	++	**76**	L3	-	+	-	-	**122**	P3	+	-	-	-
31	G2	+	+	++	++	**77**	L4	+	++	+	+	**123**	P4	+	+	++	+++
32	G3	++	+	++	++	**78**	L5	++	++	-	-	**124**	P5	+	-	++	+
33	G4	-	+	-	-	**79**	L6	+	++	+++	++++	**125**	P6	+	++	+++	+++
34	H1	-	-	+	-	**80**	L7	++	++	-	-	**126**	P7	+	++	++	+
35	H2	+	++	++	++	**81**	L8	+	++	+	+	**127**	P8	++	+	+	+
36	H3	++	++	+	+	**82**	L9	+	-	-	-	**128**	P9	+	-	-	-
37	H4	++	+	++	+	**83**	L10	+	++	+++	+++	**129**	P10	++	++	-	-
38	H5	-	++	-	-	**84**	L11	+	++	++	+++	**130**	P11	+	-	-	-
39	H6	++	++	+	+	**85**	M1	++	+	+	+	**131**	P12	+	+	-	-
40	H7	-	+	-	-	**86**	M2	++	+	+	+	**132**	P13	++	++	+	+
41	I1	-	+	+	+	**87**	M3	+++	+	++	++	**133**	P14	+	+	++	++
42	I2	++	++	+	+	**88**	M4	+	++	++	+	**134**	Q1	+	++	+	+
43	I3	+	+++	+++	+++	**89**	M5	-	++	++	-	**135**	Q2	+	+	++	+
44	I4	+	++	++	++	**90**	M6	+	+	+	++	**136**	Q3	+	++	++	++
45	I5	-	+	+	+	**91**	M7	+	+	+	++	**137**	Q4	+	++	+	+
46	I6	+	++	+	+	**92**	M8	++	+	+	++	**138**	Q5	++	+	+	+

Table 3.1 : 138 differentially expressed cDNA bands were identified on the differential display gel
- : no expression detected; + to +++: arbitrary measures for different levels of expression (+: weak expression; +++: very strong expression).

Among the up-regulated transcripts, three expression patterns were seen (Fig. 3.5): (i) transcripts up-regulated in the $T+3_{DD}$ group, whose expression had returned to baseline levels (i.e. levels similar to those in the $Box+3_{DD}$ group) 27 hours after contextual conditioning, suggesting a role in early stages of memory consolidation; (ii) transcripts up-regulated specifically in the $T+27_{DD}$ group, with levels in the $T+3_{DD}$ group similar to those in the $Box+3_{DD}$ control group, hinting at a role in later stages of consolidation; (iii) transcripts up-regulated 3 hours after training, with sustained or increased levels of expression 27 hours after training, indicating a potential role throughout the consolidation period.

Among the down-regulated transcripts, the three opposite expression patterns were observed (Fig. 3.5): (i) transcripts down-regulated in the $T+3_{DD}$ group, whose expression had returned to baseline levels 27 hours after training, (ii) transcripts down-regulated specifically in the $T+27_{DD}$ group, with levels in the $T+3_{DD}$ group similar to those in the $Box+3_{DD}$ group; (iii) transcripts down-regulated 3 hours after training, that remained repressed even 27 hours after training.

Expression changes triggered by contextual re-exposure

Forty six cDNA bands (33% of total number of regulated cDNAs) showed changes in their intensities following re-exposure of the animals to the training environment. These genes are therefore likely to play a role in memory reconsolidation.

Three different types of transcriptional profile were observed after memory reactivation, suggesting that three parallel processes occur in the hippocampus during reconsolidation (Fig. 3.6). First, some of the processes triggered by the conditioning were switched off after the re-exposure. Therefore, a proportion of the transcripts regulated by contextual

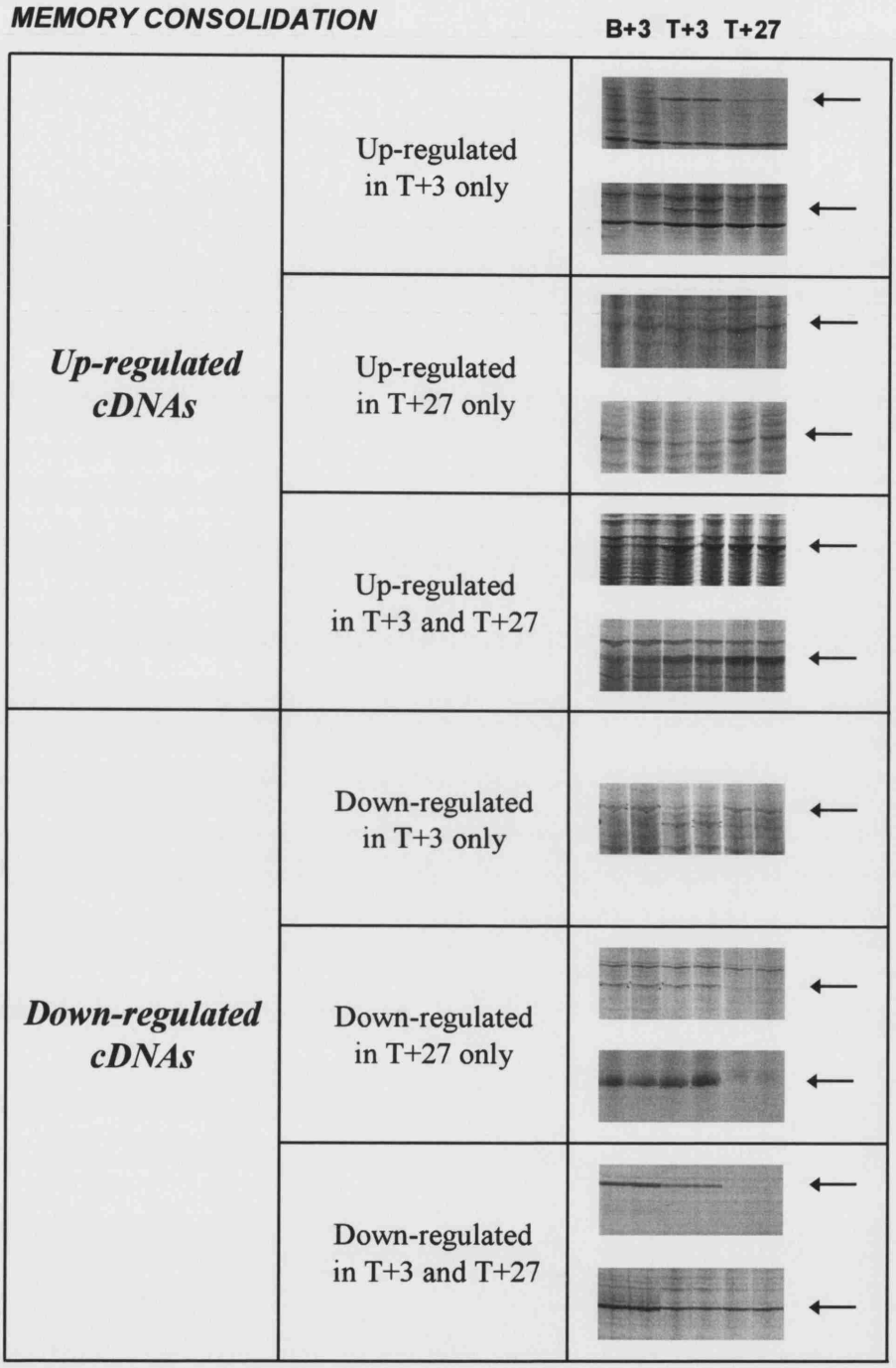

Figure 3.5 : Pattern of gene expression during memory consolidation.
Up-regulated and down-regulated transcripts were found. Three types of up-regulation were observed: up-regulated in the $T+3_{DD}$ group only, up-regulated in the $T+27_{DD}$ group only, and up-regulated in both $T+3_{DD}$ and $T+27_{DD}$ groups. Three types of down-regulation were observed: down-regulated in the $T+3_{DD}$ group only, down-regulated in the $T+27_{DD}$ group only, and down-regulated in both $T+3_{DD}$ and $T+27_{DD}$ groups.

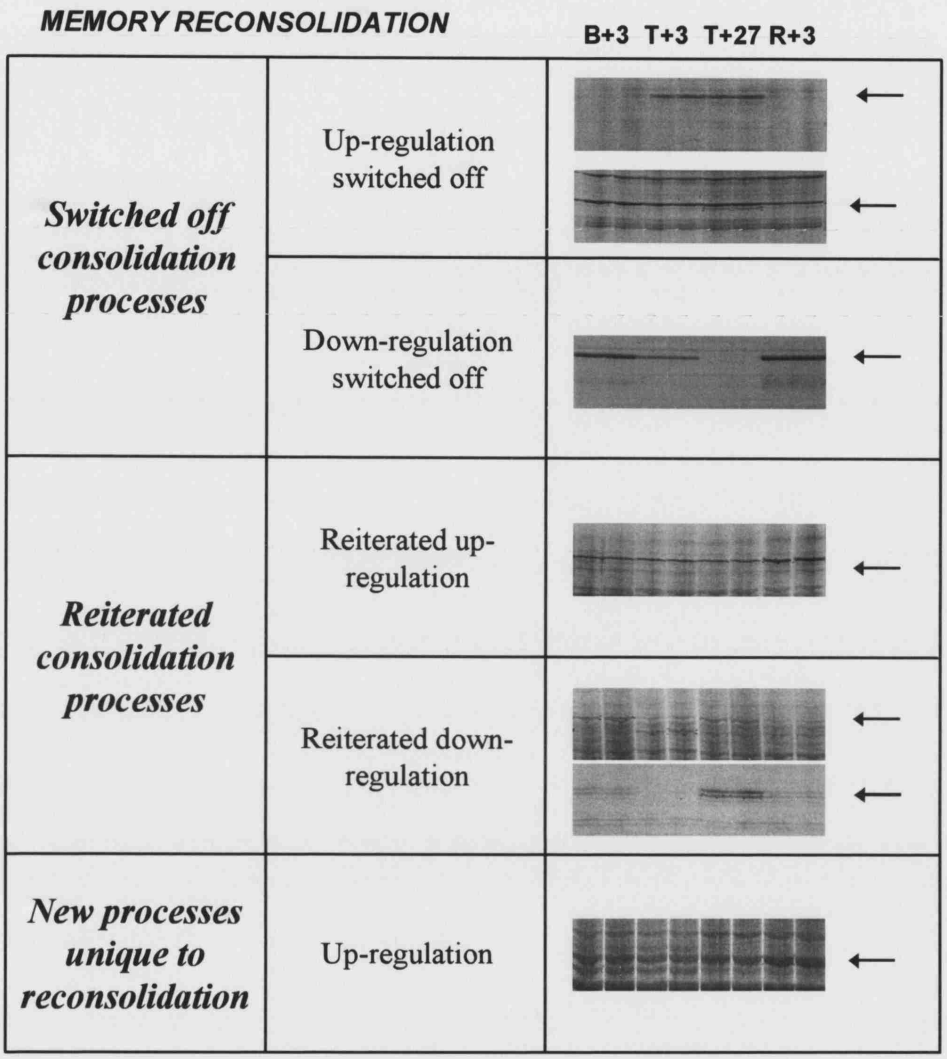

Figure 3.6 : Pattern of gene expression during memory reconsolidation. B_3: Box+3_{DD}; T_3: T+3_{DD}; Three types of process were observed: switched off consolidation processes, reiterated consolidation processes, and new processes unique to reconsolidation.

conditioning returned to baseline levels after memory reactivation. Second, some of the processes triggered by conditioning were reiterated after the re-exposure; both re-capitulated up-regulations and recapitulated down-regulations were observed. Finally new processes took place; genes that were not regulated by contextual conditioning were regulated after re-exposure.

3.1.2.3 Identity of the differentially expressed genes

Sixty five cDNAs that showed altered gene expression were eluted from the gel, PCR-amplified, and cloned into TOPO cloning vectors. These bands were chosen according to the following criteria: (i) the expression change was big as determined by visual inspection and/or (ii) the pattern of expression was rare. The clones with the correct insert length were assumed to carry the "true" positive cDNAs (see methods for detail) and two to four clones per cDNA band were sequenced. The identity of 17 cDNA bands proved difficult to determine because too many different candidates of the corresponding length were sub-cloned during the TOPO cloning, making it impossible to determine which of the clones corresponded to the cDNAs seen on the gel. This is an intrinsic difficulty of differential display, that can be bypassed using a hybridisation method described elsewhere (Vogeli-Lange et al, 1996). DNA sequence analysis revealed that the remaining 47 cDNA bands corresponded to 37 different clones.

NCBI GeneBank database searching revealed that of those 37 cDNAs, 16 encoded known proteins (Table 3.2). Nineteen genes corresponded to published genomic DNA sequences or cDNA clones with unknown identity and 5 cDNAs did not share any homology to already published DNA/cDNA sequences (Table 3.3).

No	Clone	Accession Number	Description	Function	Function in L&M/ plasticity/cognition*	*in situ* signal
1	A5	X03351	Transthyretin	Signalling	Stork 2001; Levenson 2004	not tested
2	A8	X70398	P311 (homolog of rat neuronal regeneration related protein [Nrep])	Growth/ Differentiation	-	yes
3	H1	AL672089	DNA sequence from clone RP23-295D9 on chromosome X ; seems to correspond to mouse STAG2 Highly homologous to 3'end of Homo sapiens stromal antigen 2 (STAG2) mRNA (NM_006603)	Cell cycle	-	no
4	H6	AY035213	NN5H6H tumor-related protein	Unknown	-	no
5	H8	BC055066	NADH dehydrogenase 6	Metabolism	-	no**
6	I3, O2, L6	BC052164	Eph receptor A4	Signalling	Gerlai et al 1999	yes
7	J3, J7	BC048534	RIKEN cDNA A230109K23 gene, mRNA; seems to correspond to mouse Pmch (no seq. available) Homologous to rat pro-melanin-concentrating hormone (Pmch) (M29712.1)	Signalling	-	no
8	L1	AK046127	NEL-LIKE protein 1 (NELL1)	Growth/ Differentiation	-	yes
9	L3	BC063105	CCR4-NOT transcription complex, subunit 2 (NOT2)	Transcription	-	yes
10	M4	Y17106	Transposon ETn, SELH/L3A strain	N/A	-	not tested
11	N8	BC039273	Transducin (beta)-like 2	Signalling	Perez Jurado et al 1999	no
12	N11	XM_131313	Preproenkephalin 1 (Penk1)	Signalling	Simmons and Chavkin 1996	yes
13	O3	NM_010303	Guanine nucleotide binding protein, alpha 13 (Gna13)	Signalling	-	no
14	P8	AF089815	Chimeric 16S ribosomal RNA	Translation	-	not tested
15	P10	J01420	Mitochondrion, complete genome; seems to correspond to mouse cytochromeoxidase subunit 1, Homology to rat cytochrome oxidase subunit I mRNA and tRNA-Ser gene	Metabolism	Bennett 1992, 1996	yes
16	P14	NM_025613	CREBBP/EP300 inhibitory protein 1 (Cri1)	Transcription	-	yes

Table 3.2: Identified differentially expressed cDNAs corresponding to known genes
*protein or protein isoform(s) has been implicated in either L&M, plasticity and/or cognition
***in situ* signal was detected for both sense and antisense sequences and therefore the signal could not be quantified

No	Clone	Accession Number	Description	Sequence homology	*in situ* signal
17	D1	AC121902.2	BAC clone RP24-168N14 from chromosome 8 No EST match	bp 73022 to 73331	no
18	D5	AL671853.7	DNA sequence from clone RP23-275N2 on chromosome X,	bp 20640 to 21277	yes, weak
19	D6	AC084069.28	chromosome 13 clone rp23-257b17 strain C57BL/6J No EST match	bp 163322 to 163811	yes, weak
20	D9	AC103615.8	chromosome 5, clone RP24-174D16 No EST match	bp 162821 to 163282	yes, weak
21	G1	AC135115.5	BAC clone RP24-251K13 from chromosome 10 No EST match	bp 101013 to 101736	yes, weak
22	I4	AC132570.3	BAC clone RP24-531D1 from chromosome 5	bp 155051 to 155625	not tested
23	I6, I7, L4	AC132685.3 BB179111	BAC clone RP23-278G24 from chromosome 7 adult male hypothalamus cDNA	bp 161854 to 162249	not tested
24	I9	AC122026.3	Mus musculus BAC clone RP24-390D21 from chromosome 1 No EST match	bp 78050 to 78527	yes
25	J11	BX649564.3	DNA sequence from clone RP23-405A18 on chromosome 2	bp 20729 to 21369	not tested
26	J15	AC122901.4	BAC clone RP23-40D21 from chromosome 10	bp 84129 to 84393	no
27	L7	AC148334.3	BAC clone RP23-213L20 from chromosome 13	bp 15294 to 15789	not tested
28	L11, O4	AK031677.1	13 days embryo male testis cDNA	bp 1077 to 1536	yes
29	M7	AC116129.13	chromosome 1, clone RP23-98F11	bp 131622 to 131704 & 131738 to 132055	yes
30	M8	AC125047.5	BAC clone RP23-354G14 from chromosome 3 No EST match	300 bp match only: 158706 to 15900	no
31	N12	AC123853.2	BAC clone RP23-326J1 from chromosome 19	bp 53617 to 64071	no
32	P4	CD542358.1	Only one EST match: Mus musculus Embryonic Germ Cell cDNA Library clone NIA:B0239F06	N/A	no
33	P6	AC114574.8	chromosome 6, clone RP23-151F20	bp 17007 to 17664	yes
34	P11	BX322656.11	DNA sequence from clone RP23-146D13 on chromosome X No EST match	bp 96070 to 96429	yes, weak
35	Q4	AC123791.4	BAC clone RP24-304L14 from chromosome 15	bp 106230 to 106697	no*
36	Q5	AC123552.4	chromosome 7 clone RP24-396I18 No EST match	bp 126453 to 127022	yes, weak
37-41	A2, A6, I11, N7, P7	NO MATCH	No match found for either the non-redundant database or the EST searches	N/A	no for all

Table 3.3: Identified differentially expressed cDNAs corresponding to unknown genes

**in situ* signal was detected for both sense and antisense sequences and therefore the signal could not be quantified

3.1.3 Affymetrix microarray screening

3.1.3.1 Experimental groups

Using the DD technique, most transcripts found to be regulated by contextual re-exposure corresponded to EST-, genomic- or unpublished- sequences. In fact, only four cDNAs encoded known proteins. Therefore, in addition to studying the changes observed with the differential display, another screen using Affymetrix microarrays (AM) was undertaken in an attempt to find more of these re-exposure regulated transcripts. Two different experimental conditions were compared (Fig. 3.7A; each n=3): *(i)* $T+27_A$, late memory consolidation group, killed 27 h after training and *(ii)* $R+3_A$, memory re-activation group, re-exposed to the training context 24 h after training and killed 3 h after re-exposure (i.e. 27 hours after conditioning). On the re-exposure day, the $R+3_A$ group froze substantially to the context 24 hours after training (Fig. 3.7B; 56.1 ± 14.7%), indicating that the contextual memory was reactivated. This group could therefore be used to look at expression changes occurring in the hippocampus during contextual memory reconsolidation. Six U74Av2 GeneChip microarrays were used, one for each mouse (Fig 3.8). The U74Av2 chips contain probes for approximately 12 000 mouse genes (www.affymetrix.com).

3.1.3.2 Identity of the differentially expressed genes

The data obtained from the microarray experiment were analysed using two different softwares: dChip and GenesSpring. Transcripts that were found to be significantly regulated using both softwares, and which displayed a 20% or higher fold change were considered. Only Forty genes were shown to be differentially regulated, and thus cluster analysis was not performed.

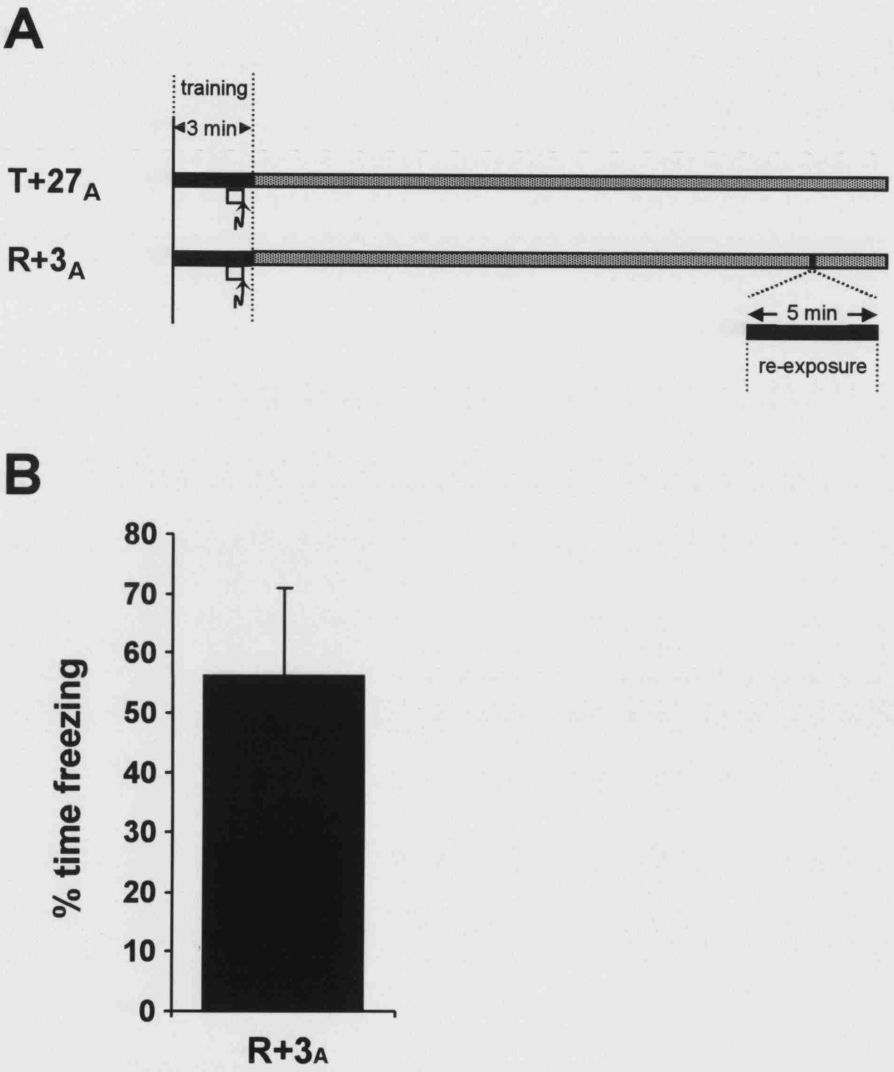

Figure 3.7 Affymetrix Microarray Experimental groups and contextual freezing scores.
(A) Experimental design to investigate changes in hippocampal mRNA expression induced by contextual re-exposure. Black boxes indicate exposure to the training context, white boxes represent a 30 s tone, the arrow symbolizes the foot shock and the filled boxes show the time until the animals were sacrificed. The following groups were studied (n=3 each): $T+27_A$: trained and killed 27 h after training; $R+3_A$: trained, re-exposed 24 h after training and killed 3 h after re-exposure.
(B) The $R+3_A$ group froze significantly to the context 24 hours after conditioning

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Figure 3.8 Experimental summary for the Affymetrix Microarray
Total hippocampal RNA for each mouse was isolated and ds-cDNA synthesised. Then cRNA was synthesised, fragmented, and hybridised to the GeneChip. 40 candidates were found to be regulated.

Of all the genes found to be up-regulated, 16 were significantly changing according to both analytical softwares and are shown in Table 3.4. Of these, 12 corresponded to known proteins and 4 to published EST sequences.

No genes were found to be down-regulated using dChip. For down-regulated transcripts a less stringent selection procedure was therefore applied, using only GeneSpring to select positive hits. 24 transcripts were detected in this way and are listed in Table 3.5.

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No genes were found to be down-regulated using dChip. For down-regulated transcripts a less stringent selection procedure was therefore applied, using only GeneSpring to select positive hits. 24 transcripts were detected in this way and are listed in Table 3.5.

Probe set*	Accession number	Description	P-value (dChip)	P-value (GS)	Fold change	Function	Function in L&M/ plasticity/cognition**
160901_at	NM_010234	FBJ osteosarcoma oncogene (c-Fos)	0.03	0.02	2.00	Transcription	Zang et al 2002
102371_at	NM_010444	Nerve Growth Factor Inducible Gene B (NGFI-B)	0.01	0.04	1.90	Transcription	Malkani and Rosen 2000
101957_f_at	NM_007415	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase) 1 (Adprtl1)	0.03	0.04	1.51	DNA repair	Cohen-Armon et al 2004
104598_at	NM_013642	MAPK Phosphatase 1 (MKP-1)	<0.01	< 0.01	1.51	Signalling	Davis et al 2000
99532_at	NM_009427	Ttransducer of ErbB-2.1 (Tob1)	<0.01	0.01	1.40	Transcription	-
93738_at	NM_011400	solute carrier family 2 (facilitated glucose transporter), member 1 (Slc2a1)	<0.01	<0.01	1.38	Transport	-
103460_at	AK017926	RIKEN cDNA 5830413E08 gene (homolog of rat RTP801)	0.02	0.01	1.36	-	Kim et al 2003
100054_s_at	N/A	DNA segment, Chr 2, Wayne State University 81, expressed	<0.01	0.04	1.35	-	-
160785_at	XM_130033	Rho GTPase activating protein 21 (Arhgap21)	0.03	0.04	1.33	Signalling	Lamprecht et al 2002
97890_at	NM_011361	serum/glucocorticoid regulated kinase (SGK-1)	0.02	0.05	1.32	Signalling	Lee et al 2003
102727_at	NM_007540	Brain derived neurotrophic factor (BDNF)	0.04	<0.01	1.31	Signalling	Gorski 2003, Liu 2004
160103_at	NM_020575	Axotrophin (Axot)	0.02	0.02	1.28	-	-
160579_at	NM_008548	mannosidase 1, alpha (Man1a)	<0.01	0.02	1.28	Metabolism	-
99032_at	NM_009026	RAS, dexamethasone-induced 1 (Rasd1)	0.02	0.01	1.26	Signalling	Brambilla et al 1997
93471_at	AI594427	Cluster Incl AI594427	0.02	0.04	1.24	-	-
100718_at	NM_008972	prothymosin alpha (Ptma)	0.01	0.02	1.24	-	Robles et al. 2003

Table 3.4: Genes found to be up-regulated on the Affymetrix microarray

*the probe set number is the identity code given by Affymetrix for the set of probes corresponding to each individual gene represented on the chip.

**protein or protein isoform(s) has been implicated in either L&M, plasticity and/or cognition

Probe set*	Accession number	Description	P-value (GeneS	Fold change	Function	Function in L&M/ plasticity/cognition**
94952_at	NM_183029	RIKEN cDNA C330012H03 gene (homolog of human hepatocellular carcinoma autoantigen	0.003	-1.63	Cell cycle	-
99584_at	NM_007656	kangai 1 (Kai1)	0.004	-1.63	-	-
95428_at	N/A	DNA segment, Chr 1, Wayne State University 40, expressed	0.006	-1.55	-	-
99558_at	NM_016746	cyclin C (Ccnc)	0.008	-1.47	Cell cycle	Ueberham et al 2003
95345_at	NM_011627	trophoblast glycoprotein (Tpbg)	0.008	-1.58	-	-
96810_at	NM_008505	LIM domain only 2 (Lmo2)	0.008	-1.45	Transcription	Hinks et al 1997
161161_r_at	NM_008704	expressed in non-metastatic cells 1, protein (Nme1)	0.012	-1.46	-	-
103394_at	NM_008761	FXYD domain-containing ion transport regulator 5 (Fxyd5)	0.013	-1.41	Ion Channel	-
95322_g_at	NM_008313	5 hydroxytryptamine (serotonin) receptor 4 (Htr4)	0.012	-1.40	Receptor	Lamirault and Simon 2001
160994_at	XM_131689	RIKEN cDNA 4930429A08 gene	0.013	-1.40	-	-
103836_at	NM_018765	WW domain binding protein 4 (Wbp4)	0.016	-1.33	-	-
100576_at	NM_008776	platelet-activating factor acetylhydrolase, isoform 1b, alpha1 subunit (Pafah1b3)	0.016	-1.34	Metabolism	-
101887_at	NM_007428	angiotensinogen (Agt)	0.020	-1.33	Homeostasis	Reviewed by Pan, 2004
98631_g_at	NM_010941	NAD(P) dependent steroid dehydrogenase-like (Nsdhl)	0.022	-1.30	Metabolism	-
97506_at	NM_011277	ring finger protein 2 (Rnf2)	0.022	-1.32	-	-
96719_i_at	NM_013645	parvalbumin (Pvalb)	0.024	-1.27	Signalling	Brady et al 1997
92401_at	NM_008521	leukotriene C4 synthase (Ltc4s)	0.029	-1.27	-	-
104155_f_at	NM_007498	activating transcription factor 3 (Atf3)	0.031	-1.30	Transcription	-
160638_at	NM_007671	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) (Cdkn2c)	0.035	-1.26	Cell cycle	Arendt et al 1998
161987_at	NM_018863	prodynorphin (Pdyn)	0.037	-1.26	Signalling	Simmons and Chavkin 1996
161650_at	NM_008175	secretory leukocyte protease inhibitor (Slpi)	0.036	-1.24	Signalling	-
92183_at	NM_207650	dystrobrevin alpha (Dtna)	0.040	-1.23	Signalling	Numakawa et al. 2004
96804_at	NM_138747	nucleolar protein 1 (Nol1)	0.045	-1.22	-	-
97224_at	XM_131355	proline-rich nuclear receptor coactivator 1 (Pnrc1)	0.045	-1.22	Transcription	-

Table 3.5: Genes found to be down-regulated on the Affymetrix microarray

*the probe set number is the identity code given by Affymetrix for the set of probes corresponding to each individual gene rep resented on the chip.

**protein or protein isoform(s) has been implicated in either L&M, plasticity and/or cognition

3.1.4 Transcriptional changes observed with the systematic screens could not be confirmed

DD and AM screens have the inherent drawback of giving false-positive signals in particular when using tissue samples (Evans et al., 2002; Lievens et al., 2001). It is therefore essential to use an independent method to confirm the changes observed.

3.1.4.1 Differential display

Thirty four of the 41 different clones detected by differential display were studied using *in situ* hybridisation: 13 out of the 16 clones corresponding to known genes and 21 out of the 25 clones corresponding to unknown genes were studied (the sequences for clones 1 and 7 was only determined at a later time point, and clone 10 corresponded to a transposable element). The screen was performed comparing four different experimental conditions. However, because of the high number of candidates to be tested, only two out of the four conditions used in the DD experiment were compared in the confirmatory experiments. Thus, the changes were investigated by (i) comparing the two conditions with the highest expression difference and (ii) using only four coronal brain slices per condition. For 17 candidates out of the 34, the *in situ* technology failed to produce a signal and thus the changes could not be confirmed using this procedure (see Table 3.2). For the remaining 17 candidates, although a quantifiable signal could be detected using this selection procedure, none of the changes could be confirmed (Data not shown).

Studying expression in four coronal sections might not have been representative of the changes happening at the level of the whole hippocampus, and thus the inability to confirm the changes could be the result of a technical limitation. The expression of four candidates was thus investigated in whole hippocampal RNA preparations using quantitative real-time

PCR (qPCR). The candidates studied were clones I9 (EST), L1 (Nell1), L3 (Not2) and P7 (EST). These candidates were selected because of their interesting expression pattern: the up-regulation in L1 and L3 was consolidation specific, and the upregulation in I9 and P7 occurring during memory consolidation was switched off during reconsolidation. Hippocampal cDNA samples from $T+27_Q$ and $R+3_Q$ group (n = 6 each) were used for clones I9 and P7, and samples from $Naïve_Q$ and $T+3_Q$ group (n = 3 each) were used for clones L3 and L4. The $R+3_Q$ group froze substantially to the context 24 hours after training (45.5± 4.9%), indicating that a contextual memory had formed, and that this memory was reactivated. This group could therefore be used to look at expression changes occurring during memory reconsolidation. qPCRs for the four candidate genes did not show any significant change in mRNA levels (Fig 3.9). Therefore, the changes in gene expression observed on the differential display gel could not be confirmed by qPCR.

3.1.4.2 Affymetrix microarray

The expression changes of eight candidates detected with the affymetrix screen was studied using qPCRs. These eight included five up-regulated transcripts (BDNF, MKP-1, NGFI-B, SGK-1, Tob1) and three down-regulated transcripts (CyclinC, Prealbumin and 5Ht4R). These candidates were chosen according to the following criteria: (i) known protein (i.e. not EST), (ii) large fold-change and/or (iii) particular molecular function (including known function in L&M). Hippocampal cDNA samples from $T+27_Q$ and $R+3_Q$ group (n=3 for each) were used. For technical reasons, however, some of the candidates were studied by comparing $T+25_Q$ vs. $R+3_Q$, instead of the $T+27_Q$ vs. $R+3_Q$. It was assumed that there was no difference in expression levels between the $T+25_Q$ and the $T+27_Q$ groups, as it had been previously shown for other genes that this was indeed the case (data not shown). Just as for the differential display, however, none of the changes detected using an Affymetrix microarray could be confirmed by qPCR (Fig. 3.10).

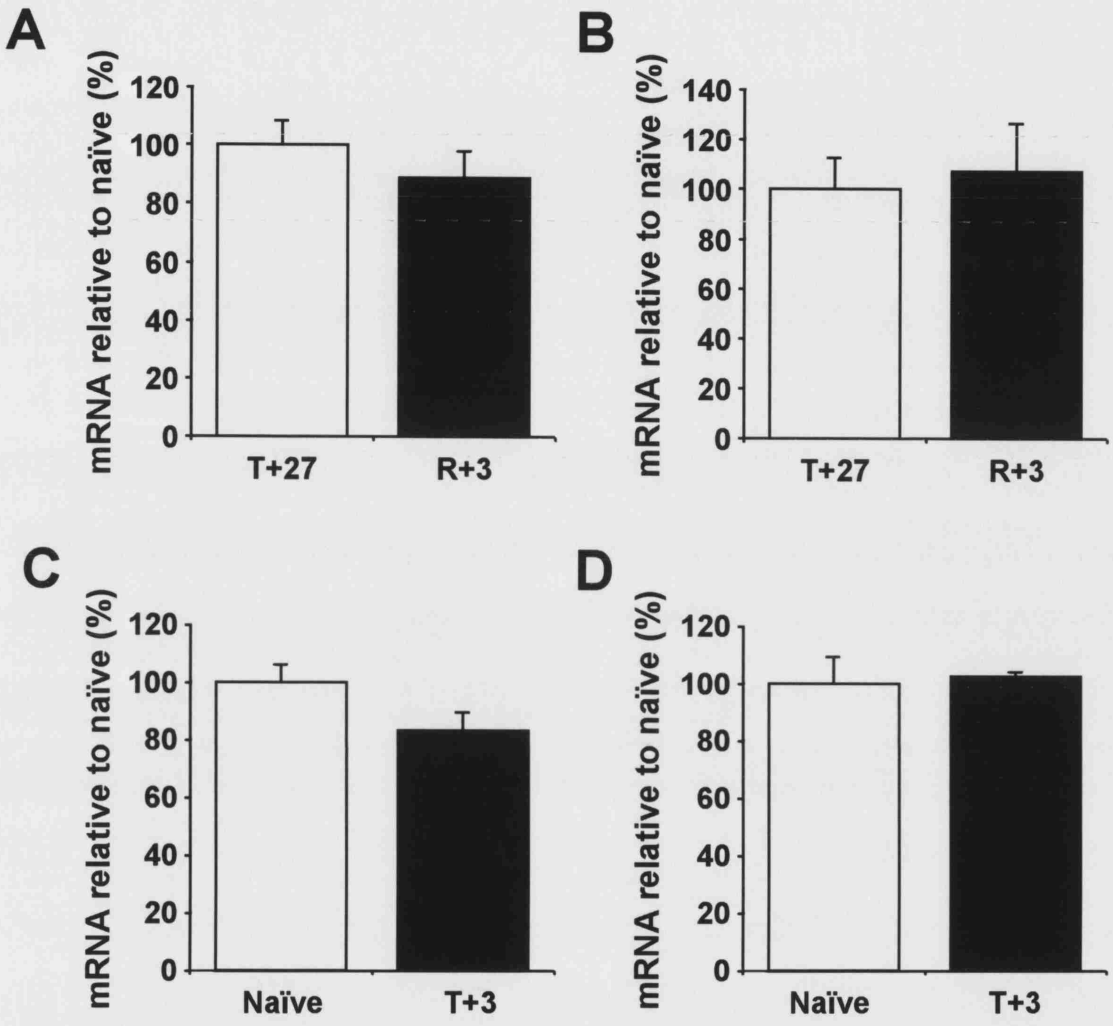


Figure 3.9: qPCR could not confirm the changes observed on the differential display
Four candidates detected with the DD were studied by qPCR and found not to be regulated during contextual memory consolidation and reconsolidation (n = 3 for Naïve and T+3; n=6 for T+27 and R+3).
(A) clone P7; **(B)** clone I9 (found to be up-regulated during reconsolidation on the DD)
(C) Nell1 (clone L3); **(D)** NOT2 (clone L4) (found to be up-regulated during consolidation on the DD)

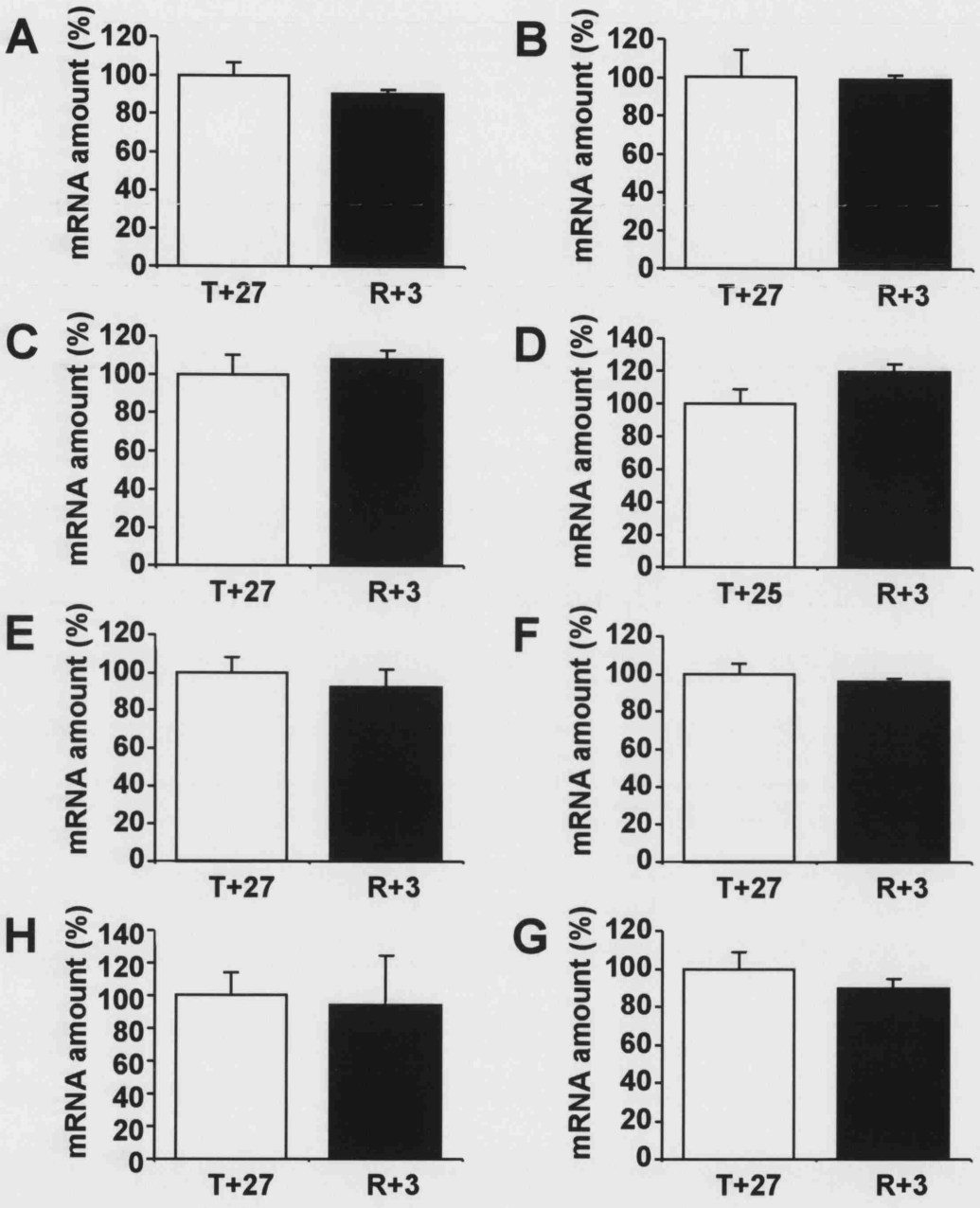


Figure 3.10: qPCR could not confirm the changes observed using the Affymetrix microarray
Eight candidates detected with the affymetrix microarray were studied by qPCR and found not to be regulated 3 h after contextual memory reactivation.
(A) BDNF; **(B)** MKP-1, **(C)** NGFI-B, **(D)** SGK-1, **(E)** TOB1 (found to be up-regulated with the AM) **(F)** Cyclin C, **(G)** Prealbumin, **(H)** 5Htr4 (found to be down-regulated with AM)

Therefore, none of the changes observed with either the DD or the AM could be confirmed. This suggests that screens such as the differential display or the Affymetrix microarrays are not suitable for detecting behaviourally induced changes in gene expression at the chosen time points. Alternatively, the magnitude of the changes might be so small, that the currently available molecular tools to confirm these changes are not powerful enough. qPCRs, for example, cannot detect changes below 20% (Bustin, 2000).

3.1.5 A more thorough investigation of the hippocampal expression of three genes: SGK-1, SGK-3 and NGFI-B

For the majority of the cDNAs detected with the differential display, the identity of the corresponding genes was not known at the time this experiment was performed. However, most of the Affymetrix candidates corresponded to known genes, and a proportion of the up-regulated transcripts were immediate-early genes (IEGs). IEGs are known to be regulated rapidly upon stimulus, hence their name. Therefore, the three hour time point used to study the differences in gene expression during memory consolidation and reconsolidation was probably not optimal to study the expression of these IEGs. An earlier time point would have been more suitable. The expression pattern of two detected IEGs, NGFI-B and SGK1, was therefore investigated at 1 hour after memory re-activation. Reactivation was performed at either 24 hours (as for the screens) or 28 days after conditioning, to study recent and remote memory reconsolidation. Changes 1 hour after contextual conditioning were also studied, so that the changes during memory consolidation and reconsolidation could be compared. In addition to these two IEGs, the expression of SGK-3, an isoform of SGK-1, was also investigated.

3.1.5.1 Experimental groups

Ten groups of mice were studied (Fig. 3.11A): *(i)* naïve (n=6), *(ii)* T+1 (n=6), early memory consolidation group, killed 1 h after training, *(iii)* T+25 (n=6), late memory consolidation group, killed 25 h after training, *(iv)* R+1 (n=6), memory reactivation group, re-exposed to the training context 24 h after training and killed 1 h after re-exposure, *(v)* box group (n=5), exposed to the training context for 3 min in the absence of foot shock and tone; returned to their home cage and killed 1 h after exposure, *(vi)* box-re-exposed group (BoxR+1) (n=6), exposed twice to the training context in the absence of foot shock and tone with an inter-exposure interval of 24 h, and killed 1 h after the second exposure, *(vii)* tone group (n=6), exposed to the training context for 3 min in the presence of the tone but in the absence of the foot shock; *(viii)* latent inhibition group (LI) (n=5), housed in the training context overnight, with water and food *ad libitum*, foot-shocked (0.75 mA) for 2 s after 16 h, and killed 1 h after the foot shock; *(ix)* $R+1_{28days}$ (n=9), remote memory reactivation group, re-exposed to the training context 28 days after training and killed 1 h after re-exposure; *(x)* $BoxR+1_{28\ days}$ (n=5), exposed twice to the training context in the absence of foot shock and tone with an inter-exposure interval of 28 days, and killed 1 h after the second exposure.

The R+1 group froze substantially to the context 24 hours after training (Fig. 3.1; 51.8 ± 5.6%), whereas the BoxR+1 group did not (0.5 ± 0.4). Similarly, animals in the $R+1_{28\ days}$ group also showed robust freezing 28 days after conditioning (50.2 ± 7.0%) whereas mice in the $BoxR+1_{28\ days}$ did not (2.3 ± 1.2%); one-way ANOVA showed a significant difference between the two groups (Fig. 3.11B; $F_{1,13} = 24.67$, $p < 0.001$). Therefore, mice in both the R+1 and $R+1_{28days}$ group re-activated the contextual fear memory, allowing these groups to be used to study the expression changes induced during memory reconsolidation. The BoxR+1 and the $BoxR+1_{28days}$ did not form a contextual fear memory, allowing this group to be used as a control for environmentally induced genes. Furthermore, the latent inhibition

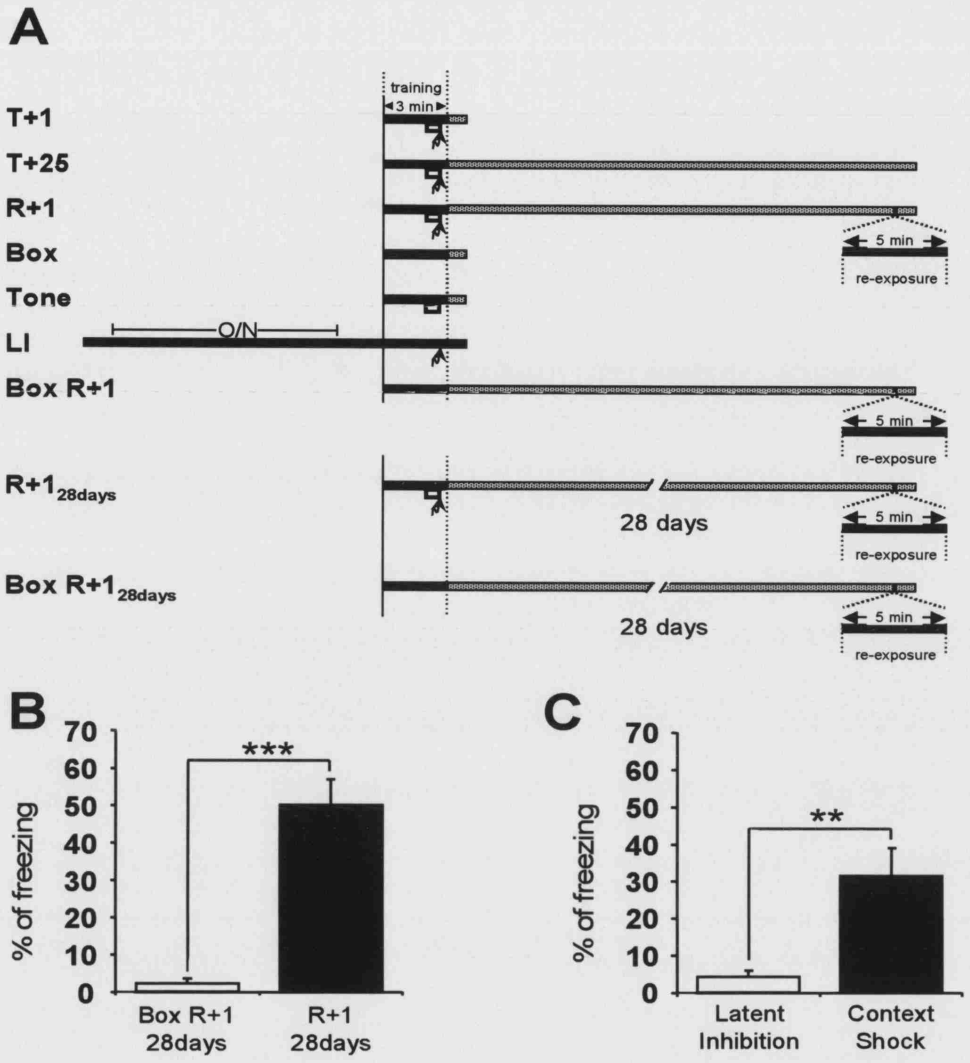


Figure 3.11 Experimental design and contextual freezing scores.
Means ± SEM, ** $p < 0.01$; *** $p < 0.001$.
(A) Experimental design to investigate changes in hippocampal mRNA expression induced by contextual conditioning. Black boxes indicate exposure to the training context, white boxes represent a 30 s tone, the arrow symbolizes the foot shock and the filled boxes show the time until the animals were sacrificed. The following groups were studied: T+1: trained and killed 1 h after training (n=6); T+25: trained and killed 25 h after training (n=6); R+1: trained, re-exposed 24 h after training and killed 1 h after re-exposure (n=6); Box: exposed to training context and killed 1 h after exposure (n=5); Tone: exposed to the training context with a 30 s tone, and killed 1 h after exposure (n=6); LI: latent inhibition control, mice were housed overnight (O/N) in the training context, shocked in the morning and killed 1 h after the shock (n=5); BoxR+1: exposed twice to the training context (interval: 24 h) and killed 1 h after the second exposure (n=5); $R+1_{28days}$: trained, re-exposed 28 days after training and killed 1 h after re-exposure (n=9); $BoxR+1_{28days}$: exposed twice to the training context (interval: 28 days) and killed 1 h after the second exposure (n=5).
(B) The freezing scores during a second exposure, 28 days after the first exposure, are shown for $BoxR+1_{28days}$ and $R+1_{28days}$. Two exposures to the context without conditioning did not evoke a freezing response ($BoxR+1_{28days}$). Contextual training induced significant freezing to context 28 days after training (R+1). ($n_{boxR+1\ 28days} = 5$, $n_{R+1\ 28days} = 9$).
(C) Housing the mice O/N in the training context, the latent inhibition protocol, significantly prevented an association between the foot shock and the context as indicated by reduced freezing contextual freezing 24 h after the foot shock (nlatent inhibition = 6, ncontext shock = 6).

(LI) protocol significantly reduced contextual freezing 24 hours after the foot shock (4.2 ± 1.7%) in comparison to freezing induced by the contextual conditioning protocol (31.4 ± 7.7%) (Fig. 3.11C; one-way ANOVA, $F_{1,10} = 12.3$, $p < 0.01$). Thus, mice trained using the LI protocol did not associate the context with the foot shock, allowing these mice to be used as a control for gene expression induced by the foot shock alone.

3.1.5.2 SGK-1 mRNA expression in the hippocampus is regulated during contextual memory consolidation and reconsolidation

To compare the expression profiles of SGK-1 during memory consolidation and reconsolidation, SGK-1 mRNA expression was first studied in four groups of mice (Naïve, T+1, T+25, R+1) (Fig. 3.12A). One-way ANOVA showed a significant difference in SGK-1 expression between the four groups ($F_{3,20} = 9.02$, $p < 0.001$). Post-hoc analysis showed that SGK-1 is significantly up-regulated one hour after contextual conditioning as compared to naïve mice ($p < 0.01$). Furthermore, 25 hours after contextual conditioning SGK-1 expression had returned to the baseline level ($p = 0.83$ for Naïve vs. T+25 and $p<0.01$ for T+1 vs. T+25). However, 25 hours after contextual conditioning SGK-1 expression was up-regulated if the memory had been reactivated 24 hours after conditioning ($p < 0.01$). This up-regulation was of similar amplitude to that observed during memory consolidation.

The Box and LI group were used to investigate whether SGK-1 expression changes during contextual memory consolidation were specific to the context-shock association or whether they were induced by the foot shock alone or context alone (Fig. 3.12B). SGK-1 expression in the Naïve, Box, LI and T+1 groups was compared (one-way ANOVA on ranks, $H_{3,18} = 13.7$, $p < 0.01$). Post-hoc analysis showed that SGK-1 expression in neither the Box nor the LI group was significantly different from SGK-1 expression in the T+1 group ($p > 0.05$ for both), even though there was a trend for higher SGK-1 expression in the T+1 group. Thus,

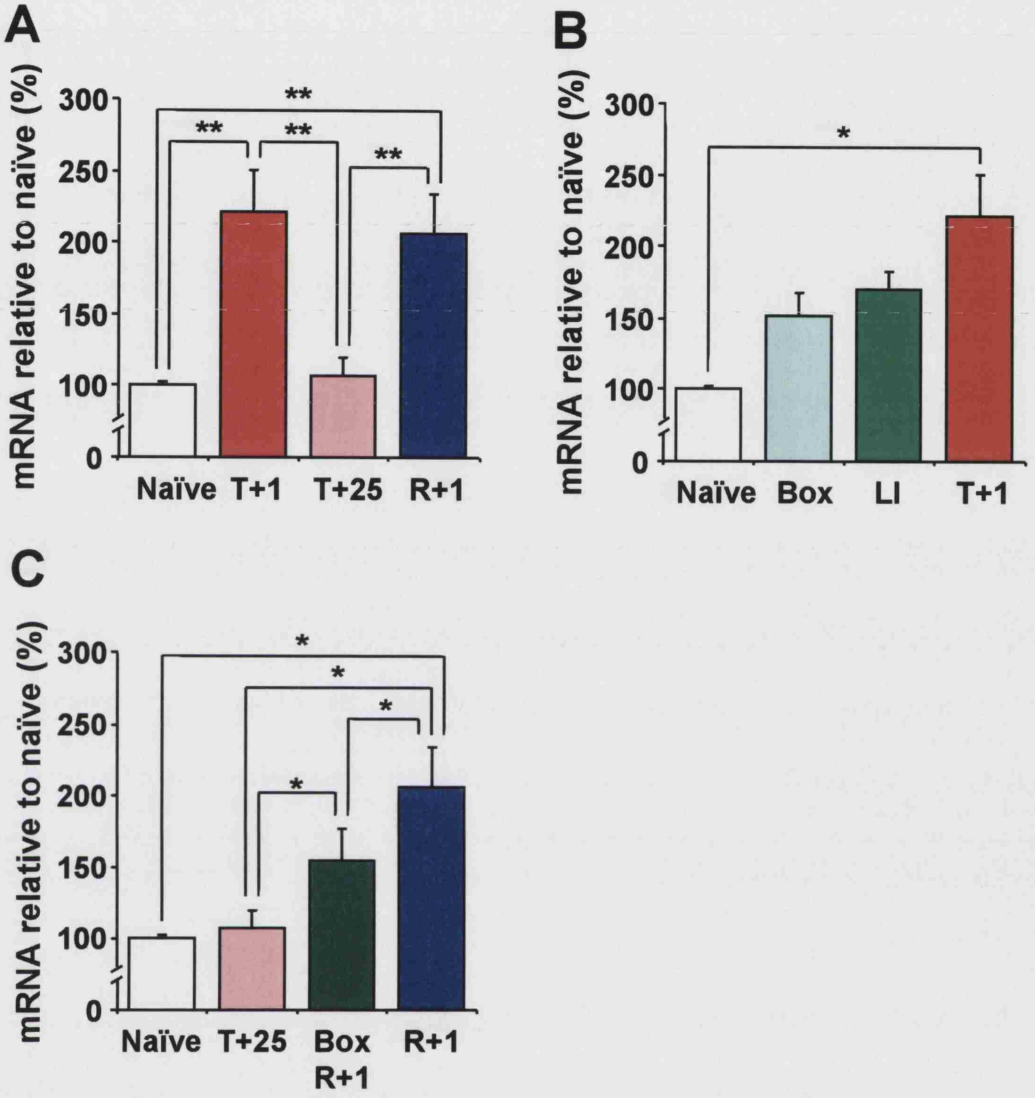


Figure 3.12 SGK-1 mRNA expression in the hippocampus is up-regulated during contextual memory consolidation and reconsolidation. Means ± SEM, ** $p < 0.01$; *** $p < 0.001$.
(A) qPCR showed that SGK-1 mRNA expression was up-regulated in the hippocampus 1 h after contextual conditioning and 1 h after contextual memory reactivation compared to the Naïve and T+25 group.
(B) SGK-1 mRNA expression in the hippocampus did not significantly differ in the Box, LI and T+1 group. Thus, the up-regulation after contextual conditioning did not appear to be specific for the learned association. [Naïve and T+1 group are the same as in A)].
(C) SGK-1 mRNA expression in the hippocampus significantly differed in the BoxR+1 vs. R+1 and in the T+25 vs. BoxR+1. Thus, up-regulation after re-exposure appeared to be induced in part by contextual memory reactivation and in part by the context alone. [Naïve, T+25 and R+1 are the same as in B)].

the up-regulation of SGK-1 expression during contextual memory consolidation was not specific to the context-shock association.

Further, the BoxR+1 group was used to study whether the up-regulation of SGK-1 expression after re-exposure was specific to memory reactivation (Fig. 3.12C). SGK-1 expression in the Naïve, T+25, BoxR+1 and R+1 groups was compared (one-way ANOVA on ranks, $H_{3,20} = 15.4$, $p < 0.01$). Post-hoc analysis revealed a significant difference between SGK- 1 expression in the BoxR+1 and R+1 groups ($p < 0.05$). Additionally, SGK-1 expression significantly differed between the T+25 and BoxR+1 groups ($p < 0.05$). Thus, up-regulation of SGK-1 expression after re-exposure appeared to be induced in part by context-shock memory reactivation and in part by the context alone.

3.1.5.3 Memory reactivation can trigger the recapitulation of an association-specific transcription: SGK-3

The hippocampal expression profile of SGK-3, an isoform of SGK-1, was also investigated during memory consolidation and reconsolidation (Fig. 3.13). A one-way ANOVA comparing Naïve, T+1, T+25, and R+1 groups showed a significant difference in SGK-3 expression between the four groups (Fig. 3.13A; $F_{3,19} = 9.7$, $p < 0.001$). Posthoc analysis revealed a significant up-regulation of SGK-3 mRNA one hour after contextual conditioning as compared to the expression in naïve mice ($p < 0.01$). Furthermore, 25 h after contextual conditioning, SGK-3 expression had returned to baseline levels ($p = 0.29$ for Naïve vs. T+25 and $p < 0.05$ for T+1 vs. T+25). However, just as for SGK-1, 25 h after contextual conditioning SGK-3 expression was up-regulated if the memory had been reactivated 24 h after conditioning ($p < 0.001$ for R+1 vs. Naïve and $p < 0.01$ for R+1 vs. T+25). This up-regulation was of similar amplitude to that observed during memory consolidation ($p = 0.17$ for T+1 vs R+1).

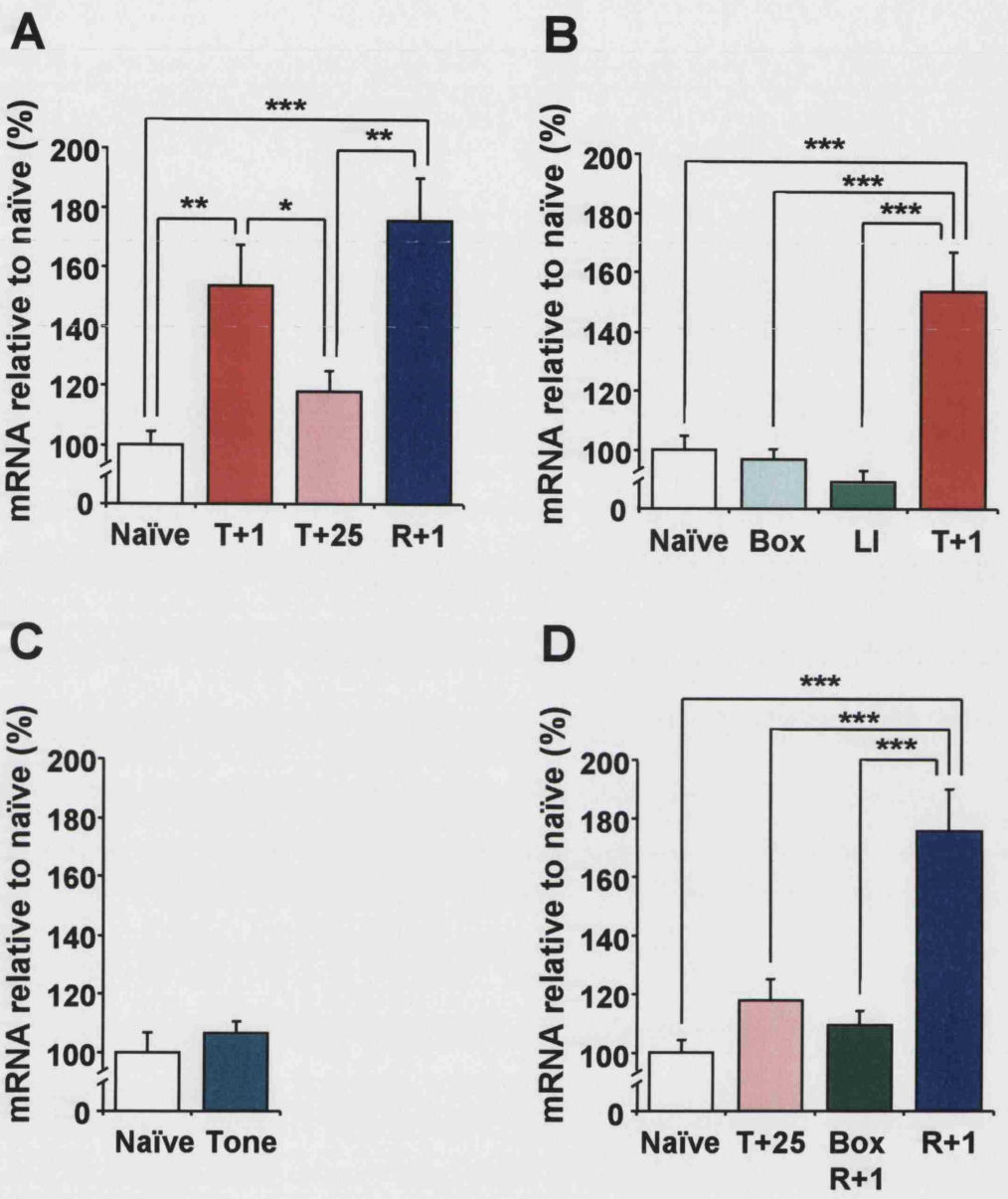


Figure 3.13 SGK-3 mRNA expression in the hippocampus is up-regulated during contextual memory consolidation and reconsolidation. Means ± SEM, * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.
(A) qPCR showed that SGK-3 mRNA expression was up-regulated in the hippocampus 1 h after contextual conditioning and 1 h after contextual memory reactivation compared to the Naïve and T+25 group.
(B) SGK-3 mRNA expression in the hippocampus in the T+1 group differed significantly from the expression in the Box and LI group; the expression in both control groups did not differ significantly from that in the Naïve group. [Naïve and T+1 group are the same as in A)].
(C) SGK-3 mRNA expression in the hippocampus did not significantly differ between the Naïve and Tone group. Thus, the up-regulation after contextual conditioning was specific for the learned association.
(D) SGK-3 mRNA expression in the hippocampus in the R+1 group differed significantly from the expression in the T+25 and BoxR+1 group. Thus, up-regulation after re-exposure was specific for contextual memory reactivation [Naïve, T+25 and R+1 are the same as in A)].

To investigate whether the expression changes during contextual memory consolidation were specific to the context-shock association or whether they were induced by the foot shock alone, the context alone, or the tone, control experiments were performed using the Box, the LI and the tone groups (Fig. 3.13B, C). SGK-3 expression differed between the Naïve, Box, LI and T+1 groups (one-way ANOVA; $F_{3,19} = 14.5$, $p < 0.001$). Post-hoc analysis showed that SGK-3 expression in the T+1 group was significantly different from the Box ($p < 0.001$) and the LI groups ($p < 0.001$). The expression in the Box ($p = 0.77$) and LI group ($p = 0.60$) was not significantly different from expression in the Naïve group. In addition, SGK-3 expression significantly differed between the Naïve and tone groups (one-way ANOVA, $F_{1,10} = 0.68$, $p = 0.43$). Thus, the up-regulation of SGK-3 expression during contextual memory consolidation was specific to the context-shock association.

Further, the BoxR+1 group was used to study whether the up-regulation of SGK-3 expression after re-exposure was specific to memory reactivation (Fig. 3.13D). SGK-3 expression in the Naïve, T+25, BoxR+1 and R+1 groups was compared (one-way ANOVA, $F_{2,19} = 15.0$, $p < 0.001$). Post-hoc analysis revealed a significant difference between SGK- 1 expression in the BoxR+1 and R+1 groups ($p < 0.001$). Additionally, the expression in the Box R+1 group was not significantly different from expression in the Naïve ($p = 0.47$) or T+25 group ($p = 0.52$). Thus, up-regulation of SGK-3 expression after re-exposure was specific to context-shock memory reactivation.

3.1.5.4 Re-activation of remote contextual memories does not trigger an up-regulation of SGK-1 or SGK-3 in the hippocampus.

The hippocampus is known to have only a temporary role in the storage of contextual fear memory. For example, lesion studies have shown that the hippocampus is not needed for remote memory (Kim and Fanselow, 1992). The transcriptional regulation of SGK-1 and

SGK-3 expression in the hippocampus was studied after the re-activation of a 28 day-old contextual fear memory (Fig. 3.14). A one-way ANOVA between Naïve, BoxR+1$_{28days}$ and R+1$_{28days}$ groups showed a significant difference in SGK-1 expression between the three groups (Fig. 3.14A; $F_{2,17} = 11.12$, $p < 0.001$). Posthoc analysis revealed a significant up-regulation of SGK-1 mRNA in the R+1$_{28days}$ group as compared to the expression in naïve mice ($p < 0.001$). However, there was also a significant up-regulation of SGK-1 in the BoxR+1$_{28days}$ group ($p < 0.05$). This up-regulation was not significantly different from that observed in the R+1$_{28days}$ group ($p = 0.15$), indicating that the up-regulation triggered by a re-exposure performed 28 days after training is induced by the context alone, and not by the reactivation of the context-shock memory. A one-way ANOVA between Naïve, BoxR+1$_{28days}$ and R+1$_{28days}$ groups showed no significant difference in SGK-3 expression between the three groups (Fig. 3.14B; $F_{2,17} = 0.27$, $p = 0.76$). Therefore, the up-regulation of SGK-1 and SGK-3, observed during reconsolidation of recent context-shock memory reactivation, is not triggered by remote context-shock memory reactivation.

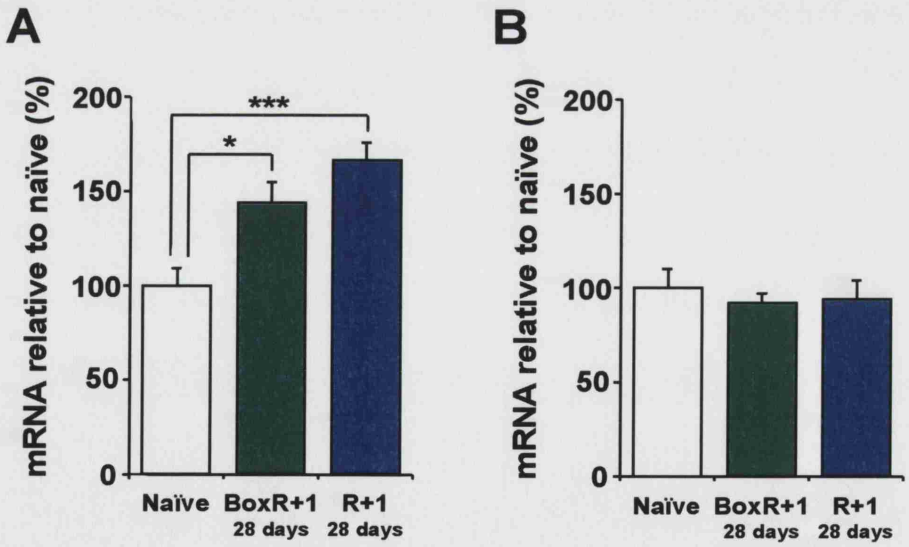


Figure 3.14 Reactivation of remote memories does not trigger an up-regulation of SGK1 or SGK3 in the hippocampus. Means ± SEM, * $p < 0.05$, ** $p < 0.01$. ($n_{naïve} = 6$, $n_{BoxR+128days} = 5$, $n_{R+128days} = 9$)
(A) SGK-1 mRNA expression in the Naïve group differed significantly from the expression in the BoxR+1$_{28days}$ and R+1$_{28days}$ group. However, SGK-1 mRNA expression in the BoxR+1$_{28days}$ and R+1$_{28days}$ groups did not differ significantly.
(B) SGK-3 mRNA expression was not significantly different in the Naïve, BoxR+1$_{28days}$ and R+1$_{28days}$ groups.

3.1.5.5 NGFI-B mRNA expression in the hippocampus is regulated during contextual memory consolidation, but not reconsolidation.

To compare the expression profiles of NGFI-B during memory consolidation and reconsolidation, NGFI-B expression was first studied in four groups of mice (Naïve, T+1, T+25, R+1) (Fig. 3.15A). A one-way ANOVA between Naïve, T+1, T+25, and R+1 groups showed a significant difference in NGFI-B expression between the four groups ($F_{3,20} = 6.67$, $p < 0.01$). Post-hoc analysis showed that NGFI-B is up-regulated one hour after contextual conditioning as compared to the expression in naïve mice ($p < 0.01$). Furthermore, 25 h after contextual conditioning, NGFI-B expression had returned to baseline levels ($p = 0.92$). Comparison of the NFGI-B expression in the R+1 vs. T+25 group ($p = 0.21$) and in the R+1 vs. Naïve group ($p = 0.36$) revealed no significant up-regulation in expression, indicating no regulation during reconsolidation.

To investigate whether the expression changes during contextual memory consolidation were specific to the learned context-shock association, control experiments were performed using the Box, the LI and the tone groups (Fig. 3.15B,C). NGFI-B expression differed between the Naïve, Box, LI and T+1 groups (one-way ANOVA; $F_{3,18} = 8.22$, $p < 0.01$). Post-hoc analysis showed that NGFI-B expression in the T+1 group was significantly different from the Box ($p < 0.01$) and the LI groups ($p < 0.05$). The expression in the Box ($p = 0.26$) and LI groups ($p = 0.24$) was not significantly different from expression in the Naïve group. In addition, NGFI-B expression did not differ between the Naïve and tone groups (one-way ANOVA, $F_{1,10} = 0.2$, $p = 0.7$). Thus, the up-regulation of NGFI-B expression during contextual memory consolidation was specific to the context-shock association.

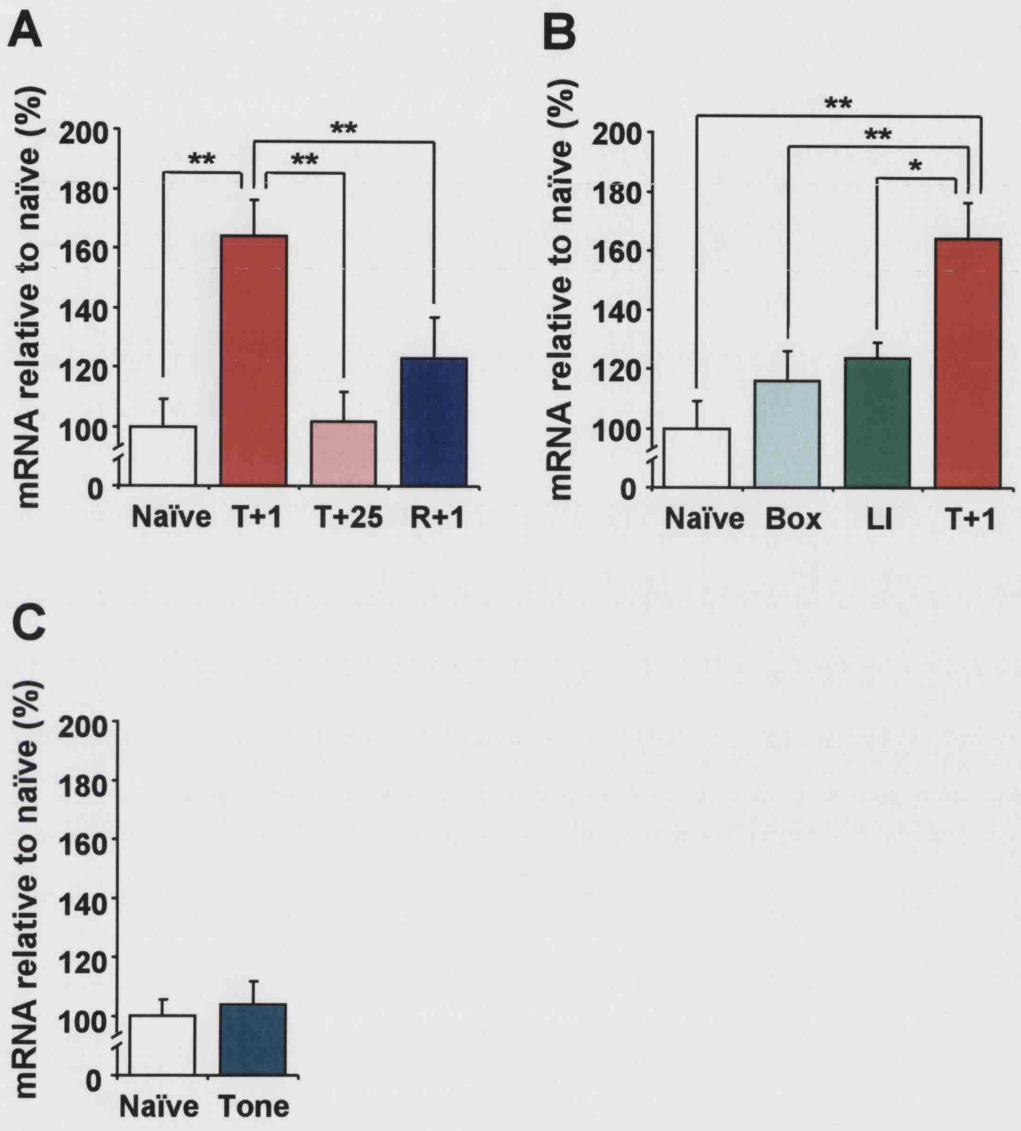


Figure 3.15 NGFI-B mRNA expression in the hippocampus is up-regulated during contextual memory consolidation but not reconsolidation. Means ± SEM, ** $p < 0.01$; *** $p < 0.001$.
(A) qPCR showed that NGFI-B mRNA expression was up-regulated in the hippocampus 1 h after contextual conditioning but not 1 h after contextual memory reactivation.
(B) NGFI-B mRNA expression in the hippocampus in the T+1 group differed significantly from expression in the Box and LI group; the expression in both control groups did not differ significantly from that in the Naïve group [Naïve, T+1 are the same as in B)].
(C) NGFI-B mRNA expression in the hippocampus did not significantly differ between the Naïve and Tone group. Thus, the up-regulation after contextual conditioning was specific for the learned association.

3.1.5.6 Up-regulation of NGFI-B mRNA expression in the hippocampus during contextual memory consolidation occurred predominantly in area CA1.

To localise the up-regulation of NFGI-B mRNA expression in the hippocampus during contextual memory consolidation, semi-quantitative *in situ* hybridisation was performed on coronal brain sections from Naïve and T+1 mice (Fig. 3.16). As previously reported (French et al., 2001), NGFI-B was almost exclusively expressed in area CA1 in the naïve mouse hippocampus (Fig. 3.16A). Contextual conditioning induced a significant three-fold CA1-specific increase of NGFI-B expression in the hippocampus one hour after training (Fig 3.16B; one-way ANOVA, $F_{1,46} = 17.03$; $p < 0.001$).

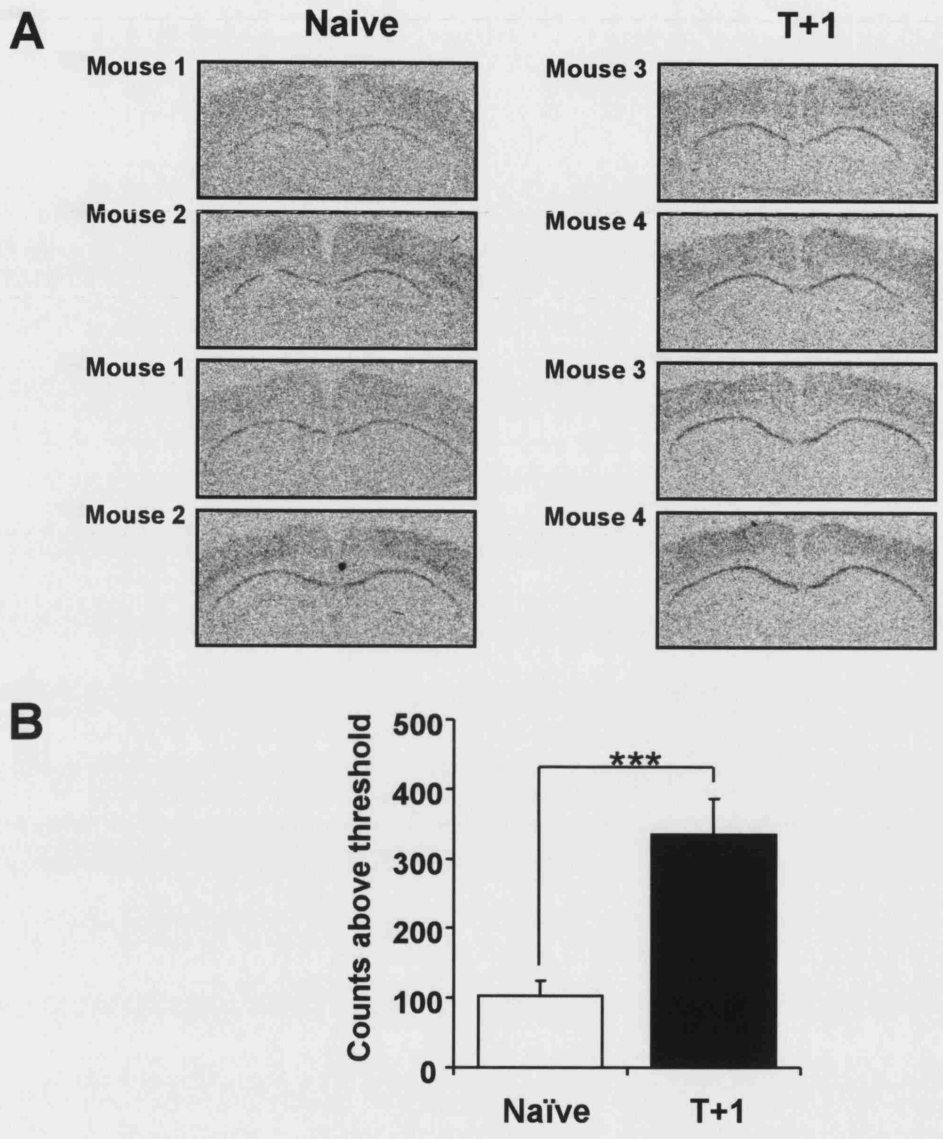
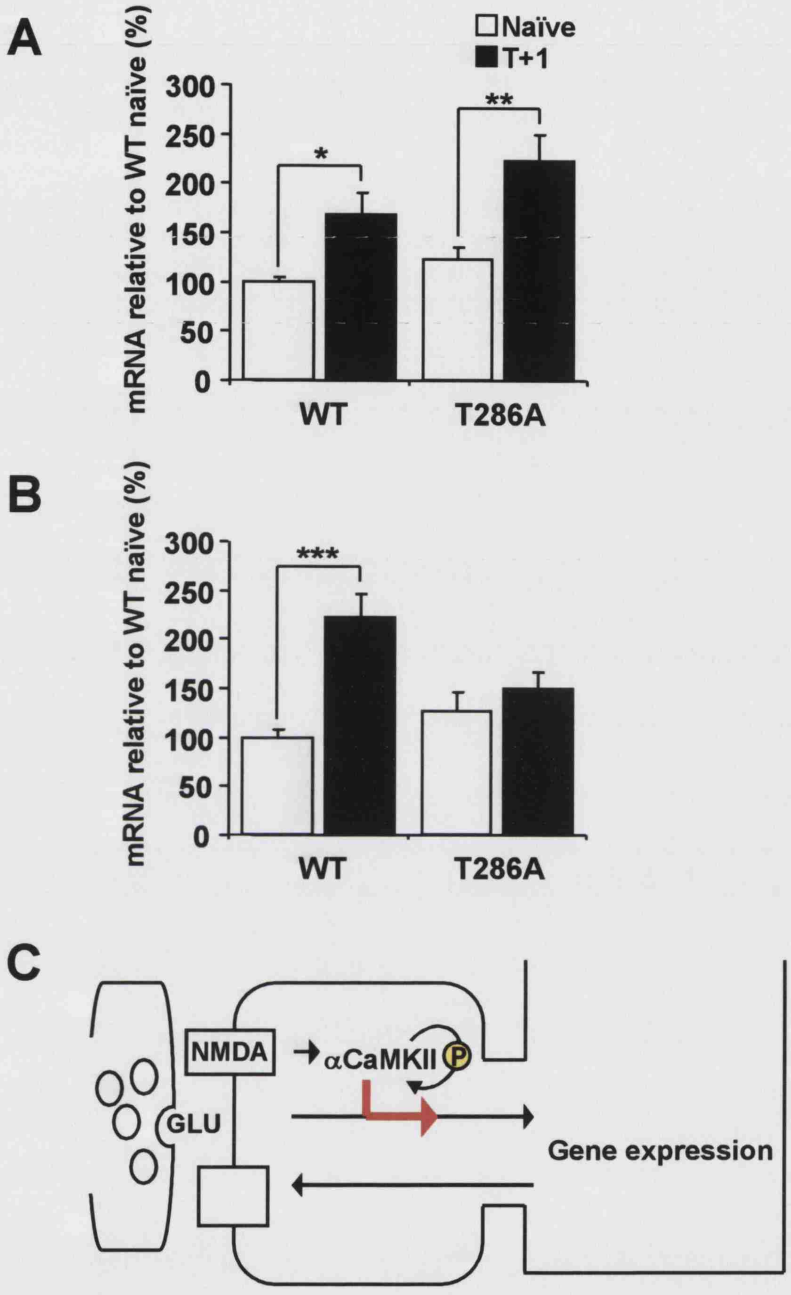


Figure 3.16 Up-regulation of NGFI-B mRNA expression in the hippocampus during memory consolidation occurred predominantly in area CA1. Means ± SEM, *** $p < 0.001$.
(A) Representative *in situ* hybridisation of coronal brain sections showing NGFI-B expression in naïve and T+1 mice (n = 2 mice per group).
(B) Semi-quantitative analysis of NGFI-B mRNA expression in area CA1 (n = 24 slices per group).

3.1.5.7 αCaMKII autophosphorylation is critical for the up-regulation of NGFI-B but not SGK1 during contextual memory consolidation

Autophosphorylation of αCaMKII is increased in the hippocampus during contextual memory consolidation (Atkins et al., 1998), and is thought to play an important part in memory storage (for review see Lisman et al., 2002). Since in the hippocampus αCaMKII is not found in the nucleus (Brocke et al., 1995; Ouyang et al., 1997), all current models assume that autophosphorylation of αCaMKII contributes to memory storage by mechanisms not involving regulation of gene expression. It is plausible, however, that the autophosphorylation of αCaMKII at the synapse provides a signal leading to changes in gene expression, a hypothesis which has not previously been tested. The T286A mutants have a targeted point mutation in the αCaMKII gene that prevents autophosphorylation, resulting in impaired in contextual memory formation (Carvalho et al., 2001) E.E. Irvine, J. Vernon and K.P. Giese, unpublished results). These mutants are therefore ideal models for examining the role of autophosphorylation of αCaMKII in *de novo* transcription during contextual memory consolidation.

First, the expression of SGK-1 in naïve and contextually conditioned mice killed 1 hour after conditioning (T+1) was studied. A two-way ANOVA for SGK-1 expression comparing Naïve $_{WT}$, T+1$_{WT}$, Naïve $_{T286A}$ and T+1$_{T286A}$ showed no training x genotype interaction (Fig. 3.17A; $F_{1,20} = 0.71$ $p = 0.41$). There was a significant effect of training ($F_{1,20} = 21.31$, $p < 0.001$), but no effect of genotype ($F_{1,20} = 4.30$, $p = 0.051$). Post-hoc analysis confirmed the earlier observation (Fig. 3.12A) that SGK-1 mRNA expression is significantly up-regulated in the hippocampus one hour after training (T+1) as compared to Naïve $_{WT}$ mice ($p < 0.05$). Furthermore, the comparison of the SGK-1 expression in the T+1$_{T286A}$ group with the Naïve$_{T286A}$ group ($p < 0.01$) revealed that the up-regulation was normal in the T286A mutants. Therefore, SGK-1 mRNA expression is up-regulated during contextual memory



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consolidation and this up-regulation does not require the autophosphorylation of αCaMKII.

Next, the expression of NGFI-B was studied (Fig. 3.17B). A two-way ANOVA for NGFI-B expression between the Naïve $_{WT}$, T+1$_{WT}$, Naïve$_{T286A}$ and T+1$_{T286A}$ groups showed that there was a significant training x genotype interaction ($F_{1,20} = 7.12$, $p < 0.05$) and a significant effect of training ($F_{1,20} = 15.36$, $p < 0.001$), but no effect of genotype ($F_{1,20} = 1.61$, $p = 0.22$). Posthoc analysis confirmed the previous finding (Fig. 3.15A) that NGFI-B expression is up-regulated in the hippocampus one hour after contextual conditioning (T+1$_{WT}$) as compared to expression in Naïve $_{WT}$ mice ($p < 0.001$). The NGFI-B expression in naïve T286A mutants did not differ significantly from expression in naïve WT mice ($p = 0.33$). However, contextual conditioning did not trigger an up-regulation of NGFI-B expression in the T286A mutants ($p = 0.39$). Thus, the up-regulation of NGFI-B expression during contextual memory consolidation required the autophosphorylation of αCaMKII, indicating that the autophosphorylation provides an up-stream signal for transcription (Fig. 3.17C).

Figure 3.17 The autophosphorylation of αCaMKII at threonine-286 regulates distinct transcriptions during contextual memory consolidation. Means ± SEM, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. (n=6 for both groups)

(A) qPCR showed that SGK-1 mRNA expression is up-regulated in the hippocampus 1 h after contextual conditioning. This up-regulation does not require the autophosphorylation of αCaMKII at threonine-286. SGK-1 expression was up-regulated 1 h after contextual conditioning in T286A mutant mice and WT littermates (n = 6 for both groups).

(B) qPCR showed that NGFI-B mRNA expression is up-regulated in the hippocampus 1 h after contextual conditioning. This up-regulation requires the autophosphorylation of αCaMKII at threonine-286. The up-regulation of NGFI-B expression 1 h after contextual conditioning was impaired in the T286A mutants in comparison to WT littermates (n = 6 for both groups).

(C) A schematic diagram showing how αCaMKII autophosphorylation might regulate transcription. Autophosphorylation of αCaMKII at the synapse (or cytosol) results in the activation of signalling pathways, which ultimately lead to activation of gene expression.

3.2 FUNCTIONAL STUDY

The association-specific up-regulation of NGFI-B mRNA during memory consolidation suggested that this transcript is important for long-term memory formation.
NGFI-B null mutant mice have been engineered (Lee et al., 1995). They develop, reproduce and appear normal (Crawford et al., 1995). To investigate the function of NGFI-B in learning and memory, the null mutant mice were tested in two hippocampus dependent tasks, contextual fear conditioning and the Morris water maze, and a hippocampus independent task, cued fear conditioning.

3.2.1 Normal contextual memory formation in NGFI-B null mutant mice

The association-specific up-regulation of NGFI-B mRNA was detected after contextual conditioning. The NGFI-B null mutants were therefore studied in this task. Mutant and WT littermates were tested either 24 hours or 28 days after training, to study both recent and remote contextual memory.

24 hours after training, the percentage freezing time did not differ between the mutants and WT mice (Mutant: 58.1 ± 4.2%, 6 females, 11 males; WT: 55.5 ± 4.0 %, 5 females, 9 males) (Fig. 3.18A; one-way ANOVA, $F_{1,30}$= 0.21 , p = 0.65). Similarly, 28 days after training, the mutants (73.8 ± 3.5%; 6 females, 5 males) and WT (65.2 ± 5.1 %; 3 females, 5 males) spend an equal amount of time freezing (Fig. 3.18B; one-way ANOVA, $F_{1,18}$ = 2.03, p = 0.17). The inability to detect a contextual memory impairment, however, does not imply that NGFI-B is not important for hippocampus-dependent memory. A memory phenotype might

have remained undetected since (i) there might be compensation for the lack of NGFI-B in the mutants as observed in the $CREB^{\alpha/\Delta}$ hypomorphic mutants (Blendy et al., 1996), (ii) there could be functional redundancy between regulated proteins during memory consolidation, (iii) the contextual conditioning task might not have adequate sensitivity to reveal the functional contribution of NGFI-B during memory consolidation. These possibilities were investigated.

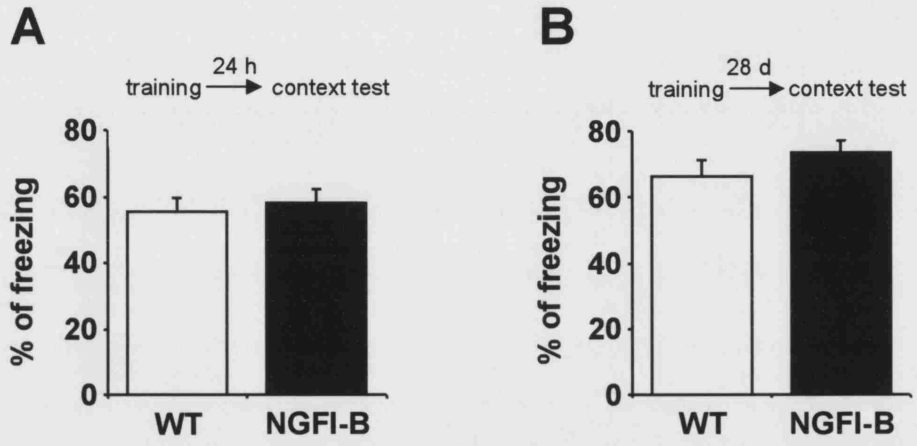

Figure 3.18: Normal contextual memory in the NGFI-B null mutants. Means ± SEM
(A) There was no significant difference in contextual freezing between the NGFI-B null mutants and WT littermates 24 h after conditioning ($n_{NGFI-B} = 16$, $n_{WT} = 14$).
(B) There was no significant difference in contextual freezing between the NGFI-B null mutants and WT littermates 28 d after conditioning ($n_{NGFI-B} = 11$, $n_{WT} = 8$).

3.2.2 Expression of Nurr1 and NOR-1 does not differ between Naïve NGFIB null mutants and WT littermates

Functional redundancy of proteins appears to be a common phenomenon among members of gene families (for review, see Tautz, 1992) and could explain the lack of contextual conditioning phenotype in the NGFI-B null mice. NGFI-B belongs to the NGFI-B subfamily of orphan nuclear receptors (for review see Maruyama et al., 1998), which

consists of three family members, NGFI-B, Nurr1 (Law et al., 1992) and NOR-1 (Ohkura et al., 1994). All three members activate transcription through a consensus sequence in the promoter of target genes (AAAGGTCA) called NGFI-B response element (NBRE) (Davis et al., 1991; Wilson et al., 1991). To test whether there is a compensatory up-regulation of Nurr1 and NOR-1 expression in the hippocampus of the NGFI-B mutants, the mRNA expression levels of both genes were studied in naïve mutant and WT mice (Fig. 3.19).

A qPCR for hippocampal Nurr1 mRNA showed no significant difference in baseline Nurr1 expression levels between the WT (n=8) and mutant (n=11) mice (Fig 3.19A; one-way ANOVA, $F_{1,18} = 1.21$, $p = 0.29$). This result is consistent with an earlier report showing no change in Nurr1 thymus mRNA levels in the mutants (Lee et al., 1995). Similarly, no altered baseline expression of NOR-1 was detected (Fig. 3.19B; one-way ANOVA, $F_{1,18} = 1.64$, $p = 0.22$). Thus, no compensation was observed in the NGFI-B mutants, and the normal fear conditioning phenotype can not be explained by higher basal levels of the two family members in the hippocampus.

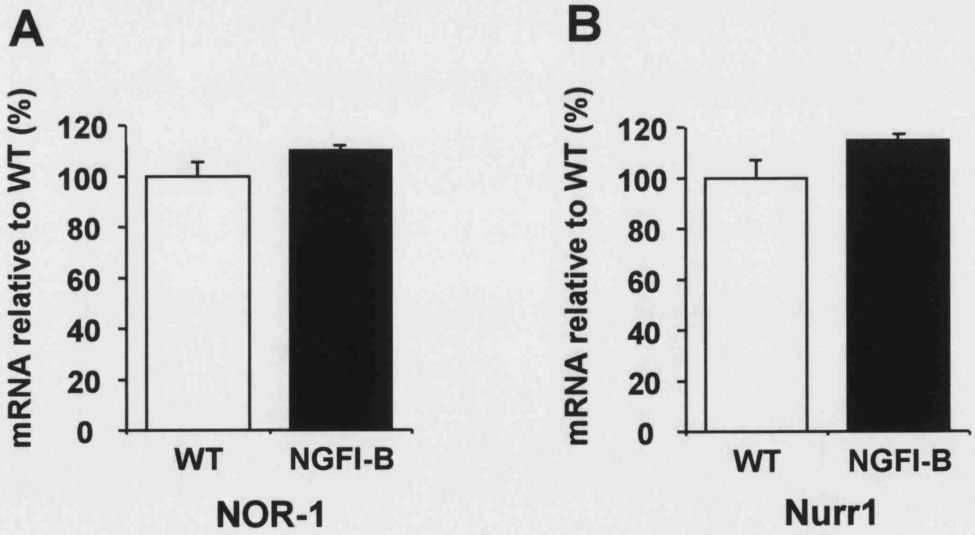

Figure 3.19 Normal baseline expression levels of NOR-1 and Nurr1 mRNA in the hippocampus of the NGFI-B null mutants. Means ± SEM ($n_{NGFI-B} = 16$, $n_{WT} = 14$).
(A) qPCR showed that NOR-1 mRNA expression was not altered in the NGFI-B mutant mice
(B) qPCR showed that Nurr1 mRNA expression was not altered in the NGFI-B mutant mice

3.2.3 Nurr1 and NOR-1 mRNA expression in the hippocampus is up-regulated during contextual memory consolidation

Contextual fear conditioning induces the phosphorylation and therefore the activation of cAMP-responsive element (CRE) binding protein (CREB) in the hippocampus, which results in CRE-mediated transcription (Impey et al., 1998). Both Nurr1 and NOR-1 contain the CRE element in their promoters (Castillo et al., 1997; Inuzuka et al., 2002). The expression of Nurr1 and NOR-1 might thus be up-regulated by contextual fear conditioning. Redundancy between up-regulated NGFI-B family members could explain the normal phenotype in the mutants. To test this hypothesis, Nurr1 and NOR-1 mRNA expression profiles were therefore investigated during contextual memory consolidation in WT Naïve, T+1 and T+25 groups (described in section 3.1.5).

A one-way ANOVA showed a significant difference in NOR-1 expression between the three groups (Fig. 3.20A, $F_{2,16} = 6.16$, $p < 0.05$). Post-hoc analysis revealed a significant up-regulation of NOR-1 mRNA in the T+25 group as compared to the expression in naïve mice ($p = 0.01$). Within the post-hoc analysis only a trend towards an up-regulation of NOR-1 in the T+1 group was observed ($p = 0.055$), but a planned comparison between the T+1 group with the Naïve group showed a significant up-regulation (one-way ANOVA, $p = 0.015$).

Similarly, a one-way ANOVA between Naïve, T+1 and T+25 groups showed a significant difference in Nurr1 expression between the three groups (Fig. 3.20B, $F_{2,16} = 5.02$, $p < 0.05$). Post-hoc analysis revealed a significant up-regulation of Nurr1 mRNA in both the T+1 and the T+25 groups as compared to the expression in the naïve mice ($p < 0.05$ for both). This result is consistent with previous findings showing an up-regulation of Nurr1 in the hippocampus 1-2 hours after contextual conditioning (Ressler et al., 2002). Therefore, a

conditioning-induced up-regulation of NOR-1 and Nurr1 could explain the undetected contextual memory impairment in NGFI-B mutants

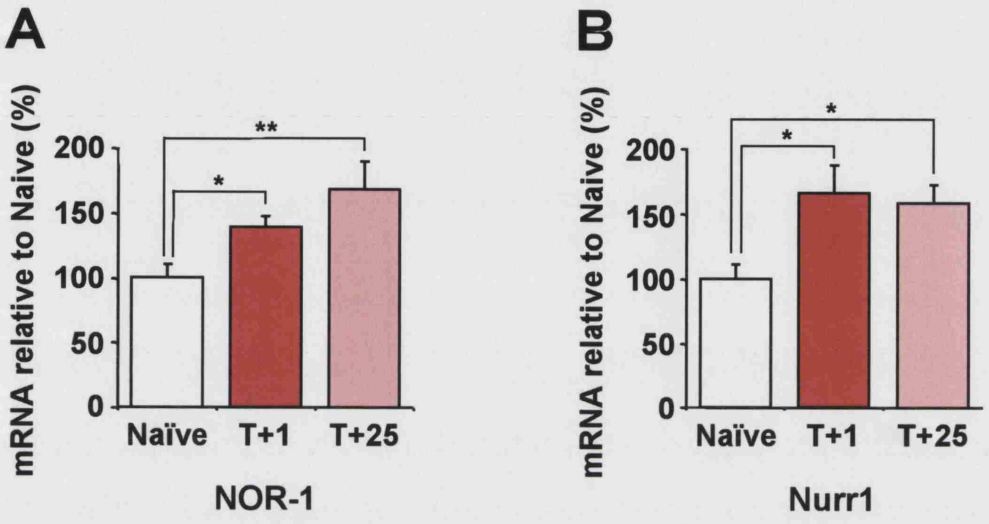

Figure 3.20 NOR-1 and Nurr1 mRNA expression in the hippocampus is up-regulated during contextual memory consolidation. Means ± SEM, * $p < 0.05$; ** $p < 0.01$. ($n_{Naïve} = 6$, $n_{T+1} = 6$, $n_{T+25} = 5$)
(A) qPCR showed that NOR-1 mRNA expression was up-regulated in the hippocampus 1 h and 25 h after contextual conditioning. The significant up-regulation 1 h after conditioning was obtained with a planned comparison between the Naïve and T+1 group.
(B) qPCR showed that Nurr1 mRNA expression was up-regulated in the hippocampus 1 h and 25 h after contextual conditioning.

3.2.4 Impaired spatial memory formation in NGFI-B null mutant mice

Contextual fear conditioning is a single trial L&M task, which might not be as sensitive as a multi-trial incremental L&M task in revealing the importance of particular genes during memory consolidation. Therefore, NGIF-B function was investigated in hippocampus-dependent spatial memory formation using the hidden-platform version of the Morris water maze (MWM). NGFI-B mice and WT littermates were trained with four training trials per

day over nine days. Our MWM setup has previously been shown to be hippocampus dependent (Angelo et al., 2003).

In the course of the nine days of training, both groups (n = 19 for mutants, n = 18 for WT) learned to locate the hidden platform as shown by a decreased latency to reach the platform (Fig. 3.21A; $F_{9,341} = 32.86$, $p < 0.001$, significant effect of training). The average time to reach the platform did not differ between mutants and control mice ($F_{1,189} = 2.59$, $p = 0.12$, no effect of genotype). Furthermore, there was no difference in swim speed or latency to reach the platform in the visible platform version of the MWM (Fig. 3.21A, one way ANOVA, $F_{1,28} = 0.056$, $p = 0.82$). In addition, the swim speed and thigmotactic behaviour was recorded during the probe trial on day 5 and 9, and was not different between NGFI-B mutant and WT littermates (Fig. 3.21B,C; one-way ANOVA, $Speed_{Day\ 5}$: $F_{1,37} = 3.57$, $p = 0.07$, $Speed_{Day\ 9}$: $F_{1,37} = 2.31$, $p = 0.14$; $Thigmotaxis_{Day\ 5}$ (on RANKS): $H_{1,37} = 2.06$, $p = 0.15$, $Thigmotaxis_{Day\ 9}$: $F_{1,37} = 0.41$, $p = 0.52$). The mutants were therefore not impaired in swimming abilities, motivation, or vision.

Analysis of the latencies to reach the platform is not a good measure for spatial memory formation, because mice can learn alternative hippocampus-independent strategies to locate the platform, such as circling in the pool at a particular distance from the pool wall. Probe trials, during which the platform is removed, are a better assessment of spatial memory. A probe trial was therefore performed on day 5. Comparison of the time spent searching in the target quadrant (TQ) where the platform was previously located with the time spent searching in the other quadrants of the pool revealed that the WT mice search selectively in the TQ (Fig. 3.22A; $F_{3,\ 71} = 12.67$, $p<0.001$; $p < 0.001$ for all TQ post-hoc comparison). This was confirmed by analysis of proximity (Fig. 3.22B; $F_{3,71} = 10.10$, $p < 0.001$; post-hoc: $p < 0.001$ for TQ vs. OP, $p = 0.001$ for TQ vs. AL, $p < 0.01$ for TQ vs. AR) and platform crossings (Fig. 3.22C; one way ANOVA on ranks; $H_{3,71} = 13.36$, $p < 0.01$; $p < 0.05$ for all

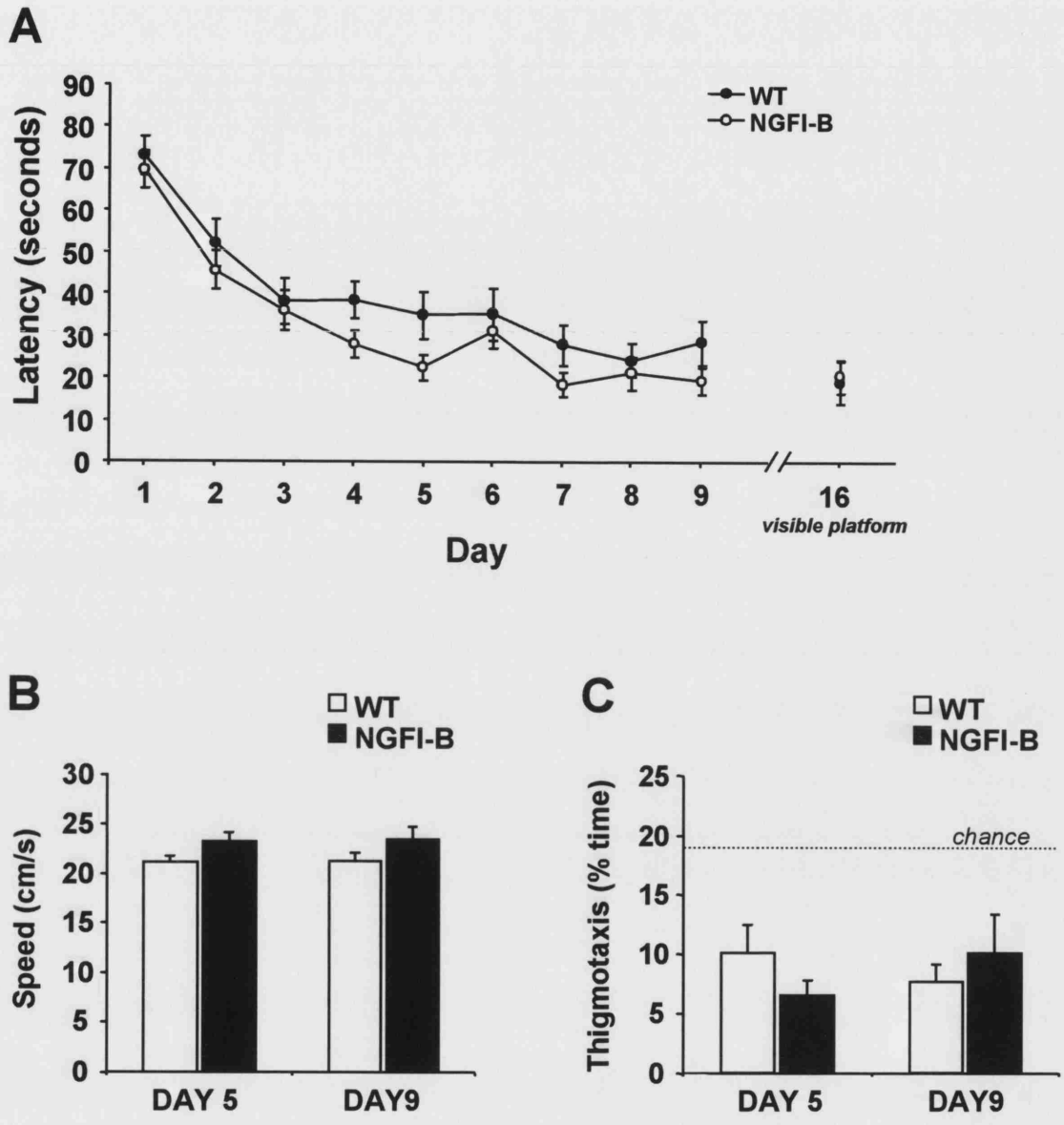

Figure 3.21 The NGFI-B mutants display normal acquisition, swimming ability, motivation & vision.
(A) The NGFI-B mutants display normal acquisition in the hidden platform version of the MWM. Both genotypes improved in their latency to find the hidden platform across the training trials; there was no difference between the genotypes. Probe trials were given at the end of the training session on day 5 and day 9. Visible platform training was performed 7 days after the end of training. The NGFI-B mutants do not show impairments in the visible platform version of the MWM. Closed circles: WT, open circles: NGFI-B null mutants
(B) Swim speed of NGFI-B mutants and their WT littermates on probe trial day 5 and 9. The NGFI-B mutants did not differ from their WT littermates.
(C) Time spent in the thigmotaxis area for NGFI-B mutants and their WT littermates, on probe trial day 5 and 9. The dotted line represents the calculated level of thigmotaxis if the mice swim randomly in the pool, which was calculated to be 19%. Both genotypes spend significantly less time in the thigmotaxis zone than expected by chance.

TQ post-hoc comparisons), which showed that the WT mice searched significantly closer to the TQ platform position and crossed that platform position significantly more than any other platforms in the pool. A typical swim path for WT mice during the probe trial is shown in Fig. 3.22D.

A one-way ANOVA for the NGFI-B mutant mice also showed a significant difference between the time spent in the quadrants (Fig. 3.22 A; $F_{3,75} = 7.33$, $p<0.001$). In contrast to WT mice, however, the NGFI-B null mutants did not search selectively in the TQ. They spent an equal amount of time in the quadrant directly adjacent left (AL) to the TQ and in the TQ ($p = 0.15$ for TQ vs. AL), but significantly more time in these two quadrants than in the two other quadrants of the pool (TQ vs. AR: $p < 0.001$; TQ vs. OP $p < 0.001$; AL vs. AR: $p <0.01$, AL vs. OP: $p < 0.05$). There was no significant difference in the time spend in the TQ between WT and mutant mice (one way ANOVA, $F_{1,36} = 0.589$, $p = 0.45$). The analysis of proximity (Fig. 3.22B; $F_{3,75} = 7.16$, $p< 0.001$) confirmed the impaired selectivity for the TQ in the NGFI-B mutants; the mice searched as close to the TQ platform position as to the AL quadrant platform position ($p = 0.35$). Furthermore, analysis of platform crossing revealed no difference in the number of crossings between the four platform positions (Fig. 3.22C; ANOVA on ranks; $H_{3,75} = 2.40$ $p = 0.49$). A typical swim path for WT mice during the probe trial is shown in Fig. 3.22D.

The mice were over-trained for another four days after the probe trial on day five, to test if the deficit in spatial memory could be overcome by prolonged training. During these additional four days of training, the latency to reach the platform significantly decreased for mutants but not WT mice ($F_{1,151} = 4.878$, $p <0.01$ significant effect of training; post-hoc for WT, $p > 0.05$; for mutants $p < 0.05$ for day 6 vs. day 7,8,9). The average time to reach the platform did not differ between mutants and WTs ($F_{1,151} = 1.78$, $p = 0.19$, no effect of genotype).

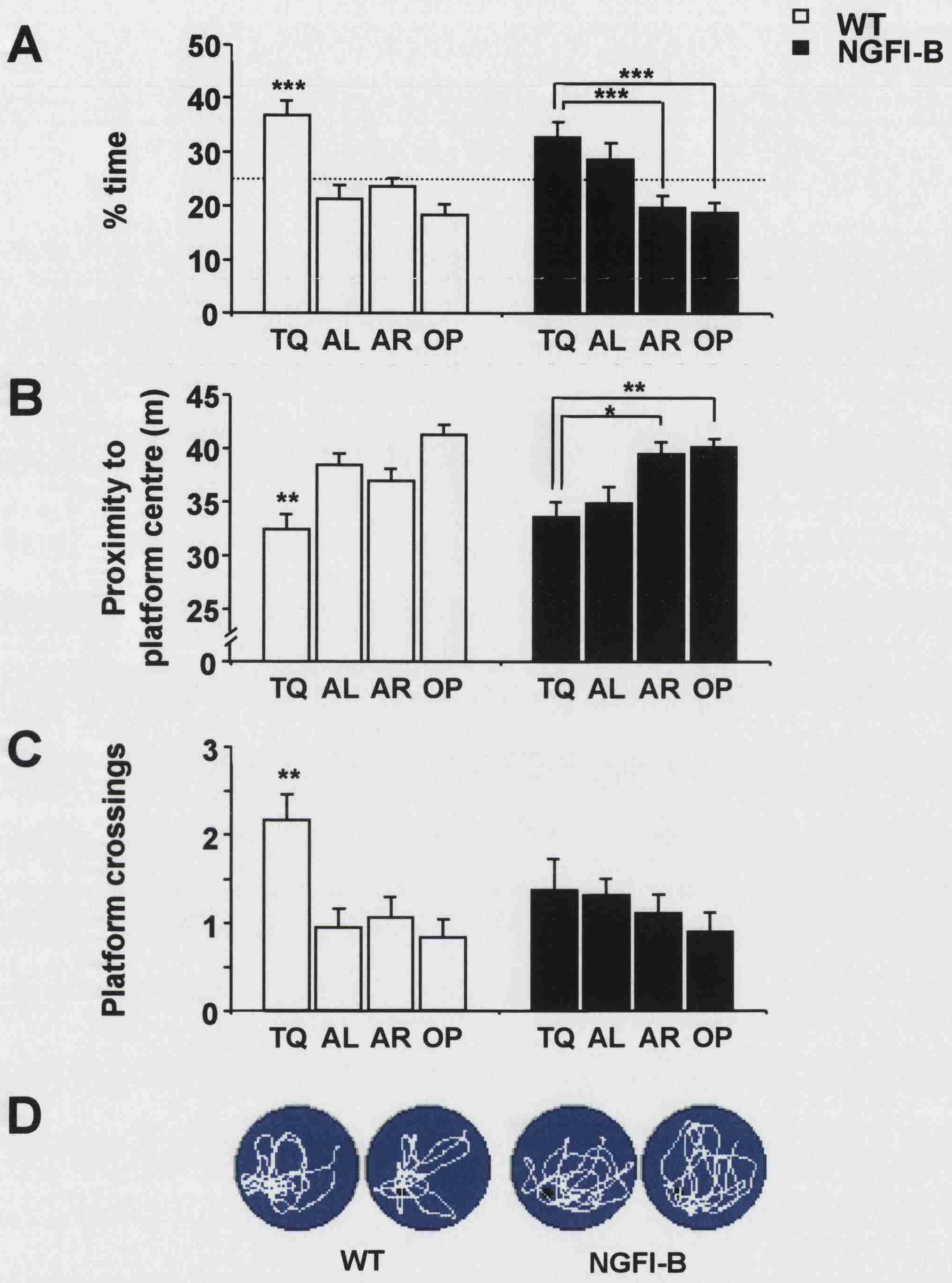

Figure 3.22 Impaired spatial memory in the NGFI-B null mutants on probe trial day 5.
Means ± SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. TQ: target quadrant; AL: adjacent left quadrant; AR: adjacent right quadrant, OP: opposite quadrant.
(A) Time spent searching in each quadrant of the pool during the probe trial. WT mice search selectively in the TQ whereas NGFI-B mutants did not.
(B) Cumulative proximity measures to each platform position. WT mice search significantly closer to the "phantom" TQ platform than to the other platform positions in the pool whereas NGFI-B mutants do not.
(C) Numbers of times each platform position was crossed. WT mice cross the target platform position significantly more than any of the other three equivalent platform positions of the pool. NGFI-B mutants crossed each platform position at equal frequency.
(D) Examples of swim paths of NGFI-B mutants and WT controls.

On the probe trial on day 9, comparison of the time spent searching in the target quadrant (TQ) where the platform was previously located with the time spent searching in the other quadrants of the pool revealed that both the WT and the NGFI-B mice search selectively in the TQ (Fig. 3.23A; WT: $F_{3, 71} = 8.84$, $p<0.001$; $p < 0.001$ for all TQ post-hoc comparisons; NGFI-B: $F_{3, 79} = 11.82$, $p<0.001$; $p < 0.001$ for all TQ post-hoc comparisons). This was confirmed by analysis of proximity (Fig. 3.23B; WT: $F_{3,71} = 3.57$, $p < 0.05$; post-hoc: $p < 0.05$ for all TQ comparisons; NGFI-B: $F_{3,79} = 5.98$, $p = 0.001$; post-hoc: $p < 0.001$ for TQ vs. OP, $p < 0.01$ for TQ vs. AL, $p< 0.05$ for TQ vs. QR) and platform crossings (Fig. 3.23C; one way ANOVA on ranks; WT: $H_{3,71} = 11.61$, $p < 0.01$; $p < 0.05$ for all TQ post-hoc comparisons; NGFI-B: $H_{3,79} = 15.562$, $p = 0.001$; $p < 0.05$ for all TQ post-hoc comparisons), which showed that both the WT and mutant mice searched significantly closer to the TQ platform position and crossed that platform position significantly more than any other platform positions in the pool.

3.2.5 Normal tone conditioning in NGFI-B null mutant mice

A previous study has shown an association-specific up-regulation of NGFI-B in the amygdala after cued conditioning (Malkani and Rosen, 2000). To test whether NGFI-B plays a role in amygdala-dependent cued fear memory consolidation, NGFI-B mutants and WT littermates were tested for cued memory either 24 hours or 28 days after training.

A two-way ANOVA comparing freezing 24 hours after training in WT and NGFI-B mutant mice before and during the CS showed a significant effect of cue (Fig. 3.24A; $F_{1,59} = 94.6$, $p < 0.001$), but no effect of genotype ($F_{1,59} = 1.181$, $p = 0.29$). There was a significant effect of training x genotype interaction ($F_{1,59} = 4.67$ $p <0.05$). Post-hoc analysis revealed a significantly higher CS freezing as compared to Pre-CS freezing in both WT and NGFI-B

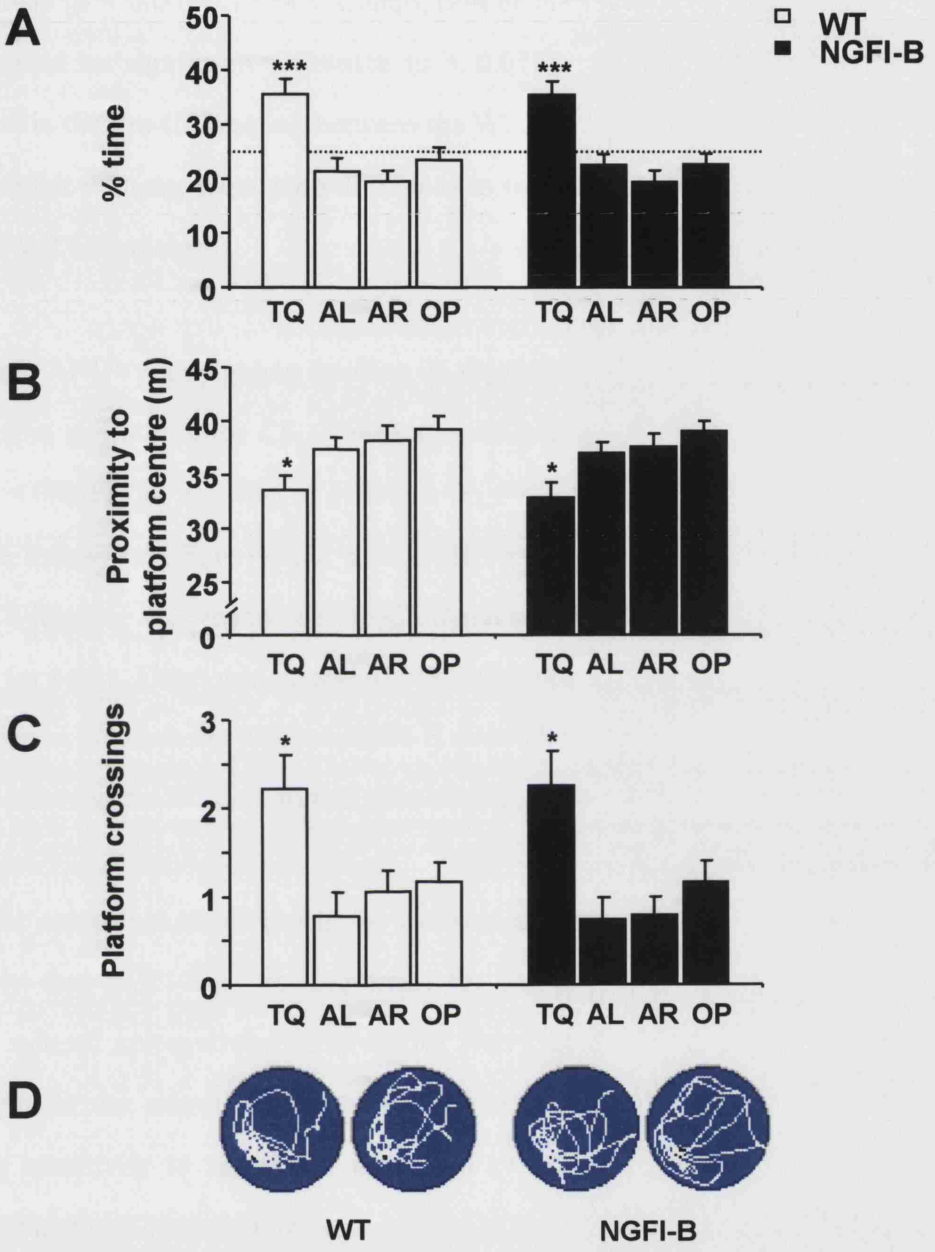

Figure 3.23 Overtraining rescues the spatial deficit in the NGFI-B null mutants tested on probe trial day 9. Means ± SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
TQ: target quadrant; AL: adjacent left quadrant; AR: adjacent right quadrant, OP: opposite quadrant.
(A) Time spent searching in each quadrant of the pool during the probe trial. Both genotypes search selectively in the TQ.
(B) Cumulative proximity measures to each platform position. Both genotypes search significantly closer to the "phantom" TQ platform than to the other three platform positions in the pool.
(C) Numbers of times each platform position was crossed. Both genotypes cross the target platform position significantly more than any of the other three equivalent platform positions of the pool.
(D) Examples of swim paths of NGFI-B mutants and WT controls.

mutant mice ($p < 0.001$ for both). Comparison of the CS freezing in the WT and NGFI-B mice showed no significant difference ($p = 0.071$). Similarly, there was no significant difference in the Pre-CS freezing between the WT and NGFI-B mice ($p = 0.79$). Therefore, 24 hours after training, there is no difference in cued memory between the mutant NGFI-B mice and WT littermates.

A two-way ANOVA comparing freezing 28 days after training in WT and NGFI-B mutant mice, before and during the CS showed a significant effect of training (Fig. 3.24B; $F_{1,37} = 122.4$, $p < 0.001$), but no effect of genotype ($F_{1,37} = 0.56$, $p = 0.47$). There was no training x genotype interaction ($F_{1,37} = 2.52$ $p = 0.13$). Post-hoc analysis revealed a significantly higher CS freezing as compared to Pre-CS freezing in both WT and NGFI-B mutant mice ($p < 0.001$ for both). There was no significant difference in the Pre-CS ($p = 0.72$) or CS ($p = 0.13$) freezing between the WT and NGFI-B mice Therefore, there was no difference in cued memory between the WT and mutant mice even 28 days after training.

Just as for contextual conditioning, the inability to detect a cued memory impairment does not imply that NGFI-B is not important for amygdala-dependent memory. Indeed, the training-induced up-regulation observed for Nurr1 and NOR-1 in the hippocampus, might also occur in the amygdala. Furthermore, the cued conditioning task might not have adequate sensitivity to reveal the functional contribution of NGFI-B during amygdala-dependent memory consolidation.

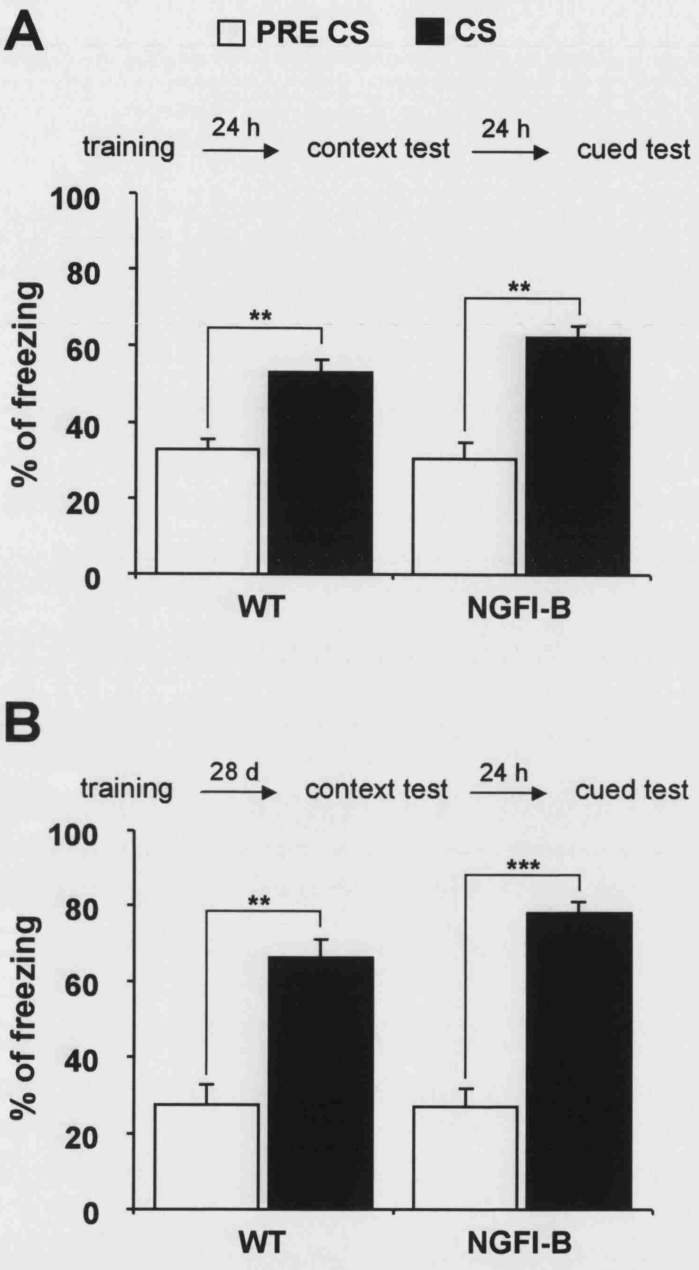

Figure 3.24 Normal cued memory in the NGFI-B null mutants.
Means ± SEM, ** $p < 0.01$, *** $p < 0.001$.
(A) There was no significant difference in cued freezing between the NGFI-B null mutants and WT littermates 24 h after conditioning ($n_{NGFI-B} = 16$, $n_{WT} = 14$).
(B) There was no significant difference in cued freezing between the NGFI-B null mutants and WT littermates 28 d after conditioning ($n_{NGFI-B} = 11$, $n_{WT} = 8$).

CHAPTER IV
DISCUSSION

4.1 SUMMARY OF THE RESULTS

Here two distinct transcriptional screens were carried out to identify genes regulated during memory consolidation and reconsolidation. Three immediate-early genes that are differentially regulated in the mouse hippocampus during consolidation and reconsolidation of contextual memory were identified: SGK1, SGK3 and NGFI-B.

The expression of all three genes was up-regulated during contextual memory consolidation. The up-regulation of SGK3 and NGFI-B expression was specific to the context-shock association, whereas SGK1 was up-regulated by the context and the shock, but not the association.

By comparing the expression pattern of SGK1 and SGK3 during consolidation and reconsolidation, it was demonstrated that some of the transcriptional events occurring during consolidation can be recapitulated during reconsolidation. It was also shown that expression changes elicited by a context-shock association during consolidation can subsequently be recapitulated during reconsolidation, and that the transcriptional changes induced by retrieval depend on the remoteness of the memory.

On the other hand, NGFI-B was shown to be regulated during consolidation but not reconsolidation. This consolidation-specific regulation in the hippocampus was localised area CA1 and requires αCaMKII autophosphorylation. Furthermore, NGFI-B was shown to be essential for hippocampus-dependent memory formation.

The following discussion is divided into three parts. First, the failure to confirm the systematic screens will be examined. Then, the molecular events involved in memory consolidation will be considered. Finally, the mechanisms underlying memory reconsolidation will be discussed.

4.2 EXPLAINING THE FAILURE OF THE TRANSCRIPTIONAL SCREENS IN THE STUDY OF LEARNING-INDUCED EXPRESSION CHANGES

In this study, two transcriptional screens (DD and AM) were used in an attempt to compare transcriptional profiles during contextual memory consolidation and reconsolidation, and to identify new genes involved in LTM formation. Although the expression patterns and the candidate genes obtained with both screens were interesting, the changes observed could not be confirmed using other methods. There are potential technical and biological reasons for this failure.

4.2.1 Technical explanation

For both the DD and the AM technology, false positives have been reported to make up a significant proportion of the differentially expressed genes when using tissue samples (Evans et al., 2002; Lievens et al., 2001). It is necessary to confirm the results obtained using another method, such as *in situ* hybridisations or qPCRs.

In situ hybridisations were used in an attempt to confirm the results obtained with the DD. However, for none of the 17 candidates for which a signal could be detected, could the changes be confirmed. It is possible that all 17 were false positive changes. Or else, it may be that the designed confirmatory experiment was not sensitive enough to detect the changes. Indeed, the expression of the genes was studied using only four coronal brain slices per condition. Four candidates were also investigated in whole-hippocampus RNA preparations using qPCRs. Yet the expression pattern for these genes could still not be confirmed, despite the larger and more representative amount of hippocampal RNA present.

qPCRs were also used to try to confirm eight candidate genes detected using the AM. However, just as for the DD, none of the changes could be confirmed.

It is still plausible that the expression changes observed using both the DD and the AM were real, but that the techniques used to confirm them were not sensitive enough. Alternatively, the proportion of false positive signals might have been extremely high for both screens. The proportion of false positives is higher if changes in gene expression are small, as background noise will interfere with the signal. There might therefore be a biological explanation as to why there was such a high proportion of false positive changes: the changes in gene expression might have been very small between the samples studied. This alternative is considered below.

4.2.2 Biological explanation

The 3 hour time point after contextual conditioning/re-exposure was chosen as the time at which gene expression was to be compared. When starting this project, it had recently been published that blocking protein synthesis 3 hours after contextual fear conditioning inhibits LTM formation (Bourtchouladze et al., 1998). Hence, at this time point, mRNAs encoding proteins essential for LTM formation must be present in the hippocampus, and should be detectable. The idea behind choosing a relatively late time point after consolidation was to avoid detecting IEGs, which usually encode transcription factors that are involved in regulating subsequent transcriptional events occurring during memory consolidation. IEGs, such as Zif268 and BDNF, had recently been shown to be regulated shortly after contextual conditioning (Hall et al., 2000). Instead the aim was to identify effector genes that would be directly involved in bringing about the changes in the cells required for information storage.

However, as the results in this study show, this time point was probably not appropriate for detecting changes underlying memory.

It is very likely that changes in gene expression nevertheless do occur 3 hours after conditioning/re-exposure, but that they are of a much lower magnitude that those occurring 30 min - 1 hour after conditioning and therefore cannot easily be detected using whole hippocampal preparations. Only a fraction of cells in the hippocampus are thought to be involved in the encoding of a memory at any one time. Therefore detecting changes occurring in these cells is likely to be difficult in the complex background of hippocampal tissue. Although in an activated neuron the transcriptional changes for the effector genes might be two-fold or higher, this change will probably remain undetected when using whole hippocampi, as most cells in such preparations are not involved in the consolidation of that memory. In contrast, changes in IEG levels are probably of much higher magnitude, and thus will be detected in whole hippocampal preparations. There is a biologically sound explanation for the difference in magnitude between the expression of IEGs and effector genes. IEGs, which in many cases are transcription factors, are usually required transiently, as they act simultaneously, to orchestrate the regulation of hundreds of genes. They are consequently required in abundance. In contrast, effector genes most probably act over a longer period of time, and are required only at the activated synapses, which represent only a minority of the total number of synapses of the activated cell. At any one time, they are therefore likely to be expressed in smaller quantities than IEGs.

It is nevertheless of great interest to the scientific community to try to understand which genes are essential for the later phases of consolidation, especially those involved in the physiological and morphological changes underlying information storage. For this, one would need to study expression profiles specifically in the cells of the hippocampus which participate in memory consolidation, and compare that profile to naïve cells. However,

isolating the hippocampal cells that are active after contextual conditioning is difficult. New techniques now available may permit such studies. One might locate activated cells using markers of neuronal activity, such as mRNAs that are regulated upon NMDA receptor-mediated signalling, to identify cells involved in memory consolidation. A transcript that would fulfil such criteria is NGFI-B. Using single cell laser capture, one could then isolate these cells and compare their expression profile to that of naïve hippocampal neurons. Such meticulous studies may nevertheless not be necessary to identify effector genes during consolidation. Microdissecting the different areas of the hippocampus, area CA1, CA2, CA3 and DG might be sufficient (e.g. Levenson et al., 2004).

4.2.3 Revised experimental design for transcriptional screens

In view of the obtained results, if one were to do such a transcriptional screen again and had more substantial financial means, one should thus proceed differently. Gene expression should be compared at least at two different time points: an early (30min to 1 h) and later (3h-4h) time point after both conditioning and after memory reactivation. The distinct hippocampal areas (CA1, CA2, CA3 and DG) should be studied in parallel, instead of using whole hippocampal preparations. If Affymetrix microarrays are used, and in the likely event that more important changes will be detected in this way, cluster analysis of the expression changes should be performed, to identify classes of genes that are regulated in the same way. If differential display technique is used, one should perform the differential display PCRs in triplicate to eliminate changes observed due to inaccurate pipetting of the cDNA into the reactions. One should also use the confirmatory experiments described by Vogeli-Lange et al. 1996) to find the cDNA corresponding to the expression change observed on the gel among the different candidates of the same length isolated from a single band.

4.3 MOLECULAR MECHANISMS UNDERLYING MEMORY CONSOLIDATION

4.3.1 SGK1, SGK3 and long-term memory formation

4.3.1.1 SGK1 and SGK3 are up-regulated during contextual memory formation

SGK1 and SGK3 are multifunctional serine/threonine kinases (for review see Firestone et al., 2003). They are expressed in all tissues so far examined, including the brain (Waldegger et al., 1997), and their activity is regulated *in vitro* by 3-phosphoinositide-dependent protein kinase (PDK) -mediated phosphorylation (Kobayashi and Cohen, 1999). *In vitro*, SGK1 and 3 have been shown to phosphorylate and thereby regulate various ion channels and transcription factors (Firestone et al., 2003).

SGK1 has recently been implicated in hippocampus-dependent L&M. Hippocampal SGK1 expression is induced by water maze training (Tsai et al., 2002) and environmental enrichment (Lee et al., 2003), a procedure known to improve hippocampus-dependent L&M (Duffy et al., 2001; Need and Giese, 2003). Furthermore, SGK1 is required for spatial learning in the water maze (Tsai et al., 2002). In agreement with the idea that SGK1 plays an important role in hippocampus-dependent L&M, hippocampal SGK1 expression was found to be up-regulated during contextual memory consolidation. This result is consistent with a recent study also showing that SGK1 is up-regulated after contextual fear conditioning (Levenson et al., 2004). The control experiments in this thesis showed that this up-regulation was not specific to the context-shock association, as there was no significant difference between the Box, LI and T+1 groups. Furthermore, the up-regulation in the T+1

group was equal to the summed up-regulation in the Box and LI groups (51% for Box, 70% for LI and 121% for T+1), indicating no association-specific increment in SGK1 up-regulation in the T+1 group. This result does not indicate, however, that SGK1 is not engaged in memory consolidation. For example, SGK1 might be involved in establishing the contextual representation within which the association takes place (Rudy et al., 2002).

These studies are the first to implicate SGK3 in L&M. Hippocampal SGK3 expression was found to be transiently up-regulated during contextual fear memory consolidation. The up-regulation was specific to the context-shock association.

4.3.1.2 Potential contributions of SGK1 and SGK3 to long-term memory

SGK1 and SGK3 have both been shown to increase the current amplitude of the *Shaker*-related K_v1 potassium channels (Gamper et al., 2002b; Gamper et al., 2002a; Warntges et al., 2002). It is therefore conceivable that during memory consolidation, SGK1 and SGK3 regulate the activity of voltage-gated potassium channels and hence neuronal excitability, a putative L&M mechanism (Giese et al., 2001; Vernon and Giese, 2004).

In neurons, the activity of potassium channels can be modulated by protein kinases and phosphatases. Potassium currents govern the onset and duration of excitations, and thus modulation of potassium channels might contribute to L&M. It may do so in three ways. First, modulating potassium channel activity might directly contribute to altering synaptic plasticity. For example, activation of potassium channels at the presynaptic terminals, leading to increased K^+ current, would result in narrowing of action potentials and reduced neurotransmitter release, thereby decreasing synaptic efficacy. Second, the modulation of potassium channels may act on the induction of synaptic plasticity. For example, A-type potassium channels have been shown to regulate action potential backpropagation (Hoffman

et al., 1997), a process whereby action potentials elicited in the axon of the postsynaptic cell actively back-propagate into the dendritic tree, contributing to postsynaptic depolarisation (Colbert, 2001). Increasing the potassium current in the dendrites by activating potassium channels would hinder action potential backpropagation, thus decreasing post-synaptic depolarisation, a requirement for NMDA receptor activation, important for LTP induction and LTM formation (Tsien et al., 1996). Third, the modulation of potassium current may participate in L&M in a way which does not affect synaptic plasticity. For example, inhibition of certain potassium channels reduces the slow after-hyperpolarisation (sAHP) whereas activation of the channels increases it. Changes in the sAHP would result in an altered firing pattern of the neuron, so that the cell is capable of firing action potentials at a different frequency. A change in the firing pattern of the cells might be required for L&M. Alternatively, a change in sAHP might act on the induction of synaptic plasticity. Action potentials that are closer together to each other in time would result in increased depolarisation of the dendritic tree and thus greater postsynaptic depolarisation, increasing the probability of NMDA receptor activation. Thus, activation of potassium channels might lead either to increased or reduced postsynaptic depolarisation. Depending on the types and location of the potassium channels that the SGK isoforms act upon, these kinases can either be memory enhancers or memory repressors.

Alterations of neuronal excitability induced by modulation of potassium channels are thought to be transient and are therefore important for learning, short-term memory, and/or the induction of LTM, rather than mechanisms underlying LTM maintenance and storage (e.g. Saar et al., 1998; Thompson et al., 1996). As SGK1 and SGK3 are induced during memory consolidation, these kinases, rather than contributing to learning, are more likely to directly contribute to the memory consolidation process (as the learning of the task has already occurred at the time when SGK1 and 3 are up-regulated). Learning genes (including SGKs) might nevertheless be triggered during memory consolidation. These "learning"

transcripts would not participate in any ongoing changes in synaptic plasticity. The corresponding proteins would, rather, be important for the induction of subsequent changes in synaptic plasticity that might, for example, support reconsolidation of fear memory. These learning proteins could also be important in the learning of multiple trial L&M tasks, where the lack of these transcripts would result in slow learning and delayed memory consolidation.

In contrast to a role in learning, SGK1 and SGK3 might directly participate in memory consolidation. SGK-1 has been shown *in vitro* to regulate gene expression by phosphorylating transcription factors (Brunet et al., 2001). Such a role might also be attributed to the two kinases during contextual memory consolidation.

To distinguish whether the SGKs play a role in learning or memory, one would have to study their role in a single trial L&M task, in which learning defects can be distinguished from memory impairments. If the SGK isoforms are required for LTM, then they must participate in memory consolidation rather than learning.

4.3.2 NGFI-B and long-term memory formation

4.3.2.1 NGFI-B is up-regulated during contextual memory formation

The third IEG investigated, NGFI-B, belongs to the orphan nuclear receptor family of transcription factors (for review see Maruyama et al., 1998). Orphan nuclear receptors are so called, because unlike the other members of the hormone nuclear receptor superfamily, their ligand is unknown. The classic nuclear hormone receptors are activated by lipophilic

ligands such as steroids, thyroid hormones, retinoids and vitamin D. The receptor-hormone complexes control gene expression by binding to regulatory elements on the DNA.

In the hippocampus, NGFI-B expression is up-regulated after seizure (French et al., 2001) and down-regulated by environmental enrichment (Olsson et al., 1994). Furthermore, contextual fear conditioning induces an up-regulation of NGFI-B expression in amygdala and neocortex in rats, which appears to be specific for associative learning (Malkani and Rosen, 2000).

In the present study, NGFI-B expression was found to be transiently up-regulated during contextual memory consolidation; this up-regulation was specific to the context-shock association. The hippocampal up-regulation in NGFI-B expression was confirmed using *in situ* hybridisation, and localised to area CA1. This result appears to disagree with the previous finding that NGFI-B expression is not altered in area CA1 of the hippocampus in the rat one hour after contextual fear conditioning (Malkani and Rosen, 2000). However, in the latter study, saturation of the *in situ* hybridisation signal is likely to have masked the transcriptional change.

NGFI-B expression has been shown to be regulated *in vitro* by the CaM kinase cascade (Blaeser et al., 2000; Inuzuka et al., 2002). During memory consolidation, the CaM kinase cascade induces the phosphorylation and thus the activation of CREB in the hippocampus, necessary for LTM (Kang et al., 2001; Peters et al., 2003; Wei et al., 2002). It is therefore possible that NGFI-B expression during consolidation is triggered by the activation of the CaM kinase cascade, and may be mediated by CREB.

The only other known gene, in addition to NGFI-B, whose up-regulation during contextual memory consolidation is specific to the context-shock association, and for which this up-regulation has been localised, is BDNF (Hall et al. 2000). The up-regulation in BDNF expression is also confined to area CA1. CA1 may therefore play an important role in encoding the context-shock association. An explanation as to how the context-shock association specific gene expression might be triggered specifically in area CA1 is by coincident signalling to area CA1 from two distinct pathways. Such a coincident signalling would only occur in animals that have been exposed to the context-shock pairing. In addition to receiving inputs from CA3 neurons via the Schaffer collateral (SC) pathway, pyramidal neurons in area CA1 are unusual in that they also receive direct cortical inputs from the entorhinal cortex (EC) via the temporoammonic (TA) pathway (Steward and Scoville, 1976). Signalling via the TA pathway enhances spiking in CA1 neurons, when the TA bursts are close enough in time to the SC-evoked spiking (Remondes and Schuman, 2002). Furthermore, TA input to area CA1 has recently been shown to be important for the consolidation of spatial LTM (Remondes and Schuman, 2004). Thus coincident signalling to CA1 neurons from SC and TA pathways might also be important to contextual fear memory, and might underlie encoding of context-shock association.

BDNF has been shown to be essential for contextual memory consolidation (Lee et al., 2004). As NGFI-B is also specific for the shock-context association and is up-regulated primarily in area CA1, it is very likely that NGFI-B also has an important role in LTM formation. This hypothesis was tested and the results discussed below.

4.3.2.2 NGFI-B is required for hippocampus-dependent memory formation

SGK1 contributes to hippocampus-dependent memory formation (Tsai et al., 2002), but no such function has been attributed to NGFI-B. To investigate whether NGFI-B contributes to hippocampus-dependent memory formation, NGFI-B null mutants were studied in fear conditioning and MWM. Although a contextual or cued memory impairment could not be detected, the NGFI-B null mutants were impaired in spatial memory formation on probe trial day 5, demonstrating that NGFI-B is important for hippocampus-dependent memory formation. The fact that there was no difference between the mutants and the WTs in the time spent in the TQ, and that the mutant mice did not swim randomly in the pool, suggests that the mutants are nevertheless capable of developing some sort of spatial memory, but that this memory is less accurate than that in WT mice.

NGFI-B is a transcription factor and is therefore likely to regulate the expression of effector proteins required for memory consolidation. With the mouse genome sequence now available, it is possible to identify all the genes that harbour the NGFI-B response element (NBRE) in their define promoter. Studying the function and regulation of such NBRE-regulated genes during memory consolidation is likely to advance the understanding of the molecular mechanisms underlying hippocampal LTM formation.

4.3.3 Functional redundancies and memory consolidation

An impairment in contextual memory could not be detected in the NGFI-B null mutant mice. Furthermore, although the mutants were impaired in spatial memory on the probe trial

on day five, they were nevertheless able to overcome the spatial memory deficit if trained for an additional four days.

Two NGFI-B family members, Nurr1 and NOR-1, were shown to be up-regulated after contextual conditioning. Nurr1 and NOR-1 have been shown to be functionally similar to NGFI-B (Davis et al., 1991; Wilson et al., 1991). Therefore, it is likely that members of NGFI-B family partially compensate for the lack of NGFI-B, resulting in a relatively mild impairment in hippocampal memory formation in the mutants. Such impairment in memory formation cannot be detected in the contexttual fear conditioning paradigm, but it can be detected in the Morris water maze, which might be a more sensitive task. Overtraining in such a task, however, results in normal spatial memory, suggesting that other proteins, likely Nurr1 and NOR-1, can support the formation of spatial LTM, but in a less efficient way than NGFI-B.

Developmental compensations, which may diisguise the importance of a particular protein in L&M, are commonly observed in null mutant mice (e.g. Blendy et al., 1996). A compensation of this kind was not observed in the NGFI-B mutants. However, the present thesis is the first study indicating that members of a same protein family, which are functionally redundant, are co-expressed during memory consolidation, showing that functional redundancies have to be taken into consideration when assessing the importance of particular proteins/families of protein in L&M.

To understand fully the mechanisms underlyiing memory consolidation, one might thus have to study the contribution of whole protein families. For this purpose, mice should be engineered, in which the function of more than one family member is disrupted. In the instance of the NGFI-B family, one might need to engineer mice that lack all three NGFI-B family members (NGFI-B, Nurr1 and NOR-1). Gene targeting that would result in the loss

of the proteins in every cell, however, is unlikely to produce viable mice, and although inducible region-restricted knockouts are in theory possible, in practice it will prove very difficult to apply such technology for multiple genes in the same organism. Recently, however, newer technologies to knock down gene expression, such as siRNAs and antisense oligonucleotides, have been developed. Both types of macromolecule consist of nucleotide sequences complementary to the mRNA of interest. Binding to the target mRNA blocks translation of the mRNAs into protein (Scherer and Rossi, 2003). Injection into specific structures of the brain, for instance the hippocampus, of a single siRNA or antisense oligonucleotide that recognises the conserved sequence of a gene family may render the study of gene families possible. Alternatively, one might use a mixture of siRNA blockers, each of which recognises a particular family member. In the instance of the NFGI-B family of transcription factors, one might also be able to use a dominant negative approach, in which a mutated form of the protein is introduced into the organism, resulting in the inhibition of the endogenous protein. Indeed, heterodimserisation between members of the NGFI-B family has been reported (Maira et al., 1999) and shows that a dominant negative mutation in one of the family members might be sufficient to block the function of the proteins in the whole family.

4.3.4 Evidence for the involvement of the hippocampus in mediating context-shock associations

Contextual fear memory is thought to depend on two processes: (i) the construction of a representation of the context and (ii) the formation of an association of that representation with the shock. The context representation is believed to be mediated by the hippocampus (Rudy et al., 2002), whereas there is debate as to where the context-shock association is generated and stored (see introduction, section 1.2.1).

SGK1 was found in the present study to be up-regulated by the context, suggesting that SGK1 contributes to the hippocampal representation of the context. In contrast, NGFI-B and SGK3 were specifically regulated by the context-shock association and are therefore likely to contribute to the storage of the learned association. This molecular evidence suggests that during contextual fear memory consolidation, the hippocampus is involved both in establishing a contextual representation as well as forming a context-shock memory. It is nevertheless possible that the formation of the context-shock memory also involves the amygdala, as suggested by some studies (e.g. Huff and Rudy, 2004; Malkani et al., 2004). However, some data suggest that instead of playing a role in the consolidation of the context-shock association, the amygdala rather modulates contextual fear memory formation (McGaugh, 2004; Moita et al., 2003).

4.3.5 A new role for αCaMKII autophosphorylation in contextual memory consolidation

One of the most intensively investigated signalling molecules in L&M is αCaMKII, a kinase required for hippocampal LTP and spatial learning (Elgersma et al., 2002; Giese et al., 1998; Lisman et al., 2002; Need and Giese, 2003; Silva et al., 1992a; Silva et al., 1992b). During contextual memory consolidation, the autophosphorylation of αCaMKII is increased in the hippocampus (Atkins et al, 1998). Furthermore, blocking the autophosphorylation of αCaMKII impairs normal contextual memory formation (Cavallaro et al., 2002); E.E. Irvine, J. Vernon and K.P. Giese, unpublished results).

Because αCaMKII is concentrated at the synapses and can act as a "molecular memory device", whereby activity can persist in the absence of any synaptic input (see Introduction), it has been suggested that the kinase is important in the maintenance of memory; it could

"tag" activated synapses and recruit other synaptic proteins that facilitate information storage (Lisman et al., 2002; Lisman, 2003). In the hippocampus, αCaMKII is not found in the nucleus (Brocke et al., 1995; Ouyang et al., 1997), and therefore it is generally assumed that this kinase does not regulate gene expression. It is however possible that the autophosphorylation of the kinase at the synapse (or cytosol) provides a signal leading to changes in gene expression, a hypothesis not previously tested.

Here, it was investigated whether the lack of αCaMKII autophosphorylation affects *de novo* gene expression, which is essential for consolidation and reconsolidation of contextual memory (Kida et al., 2002). CaMKII has been shown to regulate NGFI-B expression (Laabich et al., 2001), whereas no CaMKII-regulation of SGK1 expression has been described. The up-regulation of NGFI-B but not SGK1 expression during memory consolidation was found to require the autophosphorylation of αCaMKII. Therefore, the autophosphorylation is essential for a subset of the transcriptional events occurring in the hippocampus during memory consolidation. This suggests that the autophosphorylation of αCaMKII provides an upstream signal for transcription (Fig. 3.17C).

Furthermore, autophosphorylation of this kinase is required for NMDA receptor-dependent LTP at glutamatergic CA1 synapses in the hippocampus (Giese et al., 1998). Since NGFI-B is required for hippocampus-dependent memory formation, and the increase in NGFI-B expression was predominant in area CA1 and required the autophosphorylation of αCaMKII, the NGFI-B up-regulation is likely to be mediated by NMDA receptor-dependent synaptic plasticity. This idea is supported by the finding that glutamate treatment of cultured hippocampal neurons up-regulates NGFI-B expression through NMDA- but not AMPA-receptor activation (Bading et al., 1995).

In contrast with NGFI-B expression, the up-regulation of SGK1 expression does not require autophosphorylation of αCaMKII, suggesting that it does not result from an NMDA receptor-dependent synaptic plasticity. Consistent with this idea, environmental enrichment increases hippocampal SGK1 expression in an NMDA receptor-independent, but AMPA receptor-dependent manner (Lee et al., 2003)). Normal up-regulation of SGK1 expression in the behaviourally impaired T286A mutants shows that the up-regulation of SGK1 expression is not sufficient for contextual memory formation.

Taken together, these findings show that during the early phase of contextual fear memory consolidation, there are transcriptional events that depend on αCaMKII autophosphorylation, whereas others do not.. It is possible that the autophosphorylation of αCaMKII controls the transcriptional processes involved in the context-shock association, whereas the construction of the context representation involves processes independent of the autophosphorylation of αCaMKII.

4.4 MOLECULAR MECHANISMS UNDERLYING RECONSOLIDATION

4.4.1 A subset of transcriptional events is recapitulated during contextual memory reconsolidation

The up-regulation of both SGK1 and SGK3 expression was found to be recapitulated during memory reconsolidation (Fig. 3.16, 3.20). This is in agreement with two other studies showing that zif268 and c-fos, known to be up-regulated in the hippocampus during contextual memory consolidation, are also up-regulated in the hippocampus during reconsolidation (Hall et al., 2000; Hall et al., 2001; Stanciu et al., 2001; Strekalova et al., 2003). For SGK1, this re-iterated up-regulation was induced in part by the context-shock memory reactivation and in part by the context alone. For SGK3, the up-regulation was specific to context-shock memory reactivation. Therefore, as was the case for consolidation, the induction mechanisms for the two IEGs differed during reconsolidation. Nonetheless, the repeated up-regulation observed for both transcripts in the R+1 group demonstrates that at least some transcriptional events are common to both consolidation and reconsolidation.

Although processes that are context-induced during consolidation have previously been shown to be recapitulated during reconsolidation (e.g. zif268, Hall et al., 2001), this is the first report showing that a process specific to context-shock association, is recapitulated after memory reactivation. These results suggest that just as in consolidation, during reconsolidation the hippocampus is engaged in two types of memory process: one mediating contextual representations and the other mediating the context-shock association. As association-specific transcripts are very likely to contribute to memory storage, these results suggest that reconsolidation gates subsequent storage of the reactivated memory, and that

the reduced conditioned response elicited by inhibitor treatments is not solely, if at all, due to retrieval deficits as suggested by recent reports (Anokhin et al., 2002; Fischer et al., 2004; Lattal and Abel, 2004).

4.4.2 Retrieval of remote and recent memory does not trigger the same molecular events

The expression of SGK1 and SGK3 was found to be up-regulated in the hippocampus by context-shock memory reactivation induced 24 hours after training. Interestingly, the expression of SGK1 and SGK3 was not altered by context-shock memory reactivation induced 28 days after the initial training. A recent study has demonstrated that remote contextual memories are more stable than recent memories, and require a longer reactivation session in order to become sensitive to disruption by anisomycin (Suzuki et al., 2004). It is therefore possible that 28 days after training, a longer re-exposure would be required to induce the context-shock memory reactivation specific up-regulation of SGK1 and SGK3. Nonetheless, these results show that the hippocampal processes occurring during reconsolidation of contextual memory change over time.

The context alone induced an up-regulation of SGK1 after a re-exposure performed 24 hours as well as 28 days after training. Thus, in contrast to context-shock associated changes, the molecular processes that underlie reconsolidation of the memory for the context representation appear to be re-iterated independently of time.

4.4.3 At the molecular level, memory reconsolidation does not recapitulate consolidation

The expression of SGK1 and SGK3 was up-regulated during consolidation and reconsolidation of recent memories. This recapitulated SGK1 and SGK3 expression showed that the hippocampus is engaged in both the consolidation and the reconsolidation of contextual fear memory, a finding consistentt with previous studies (Debiec et al., 2002; Hall et al., 2000; Hall et al., 2001). Yet, in contrast to SGK1 and SGK3, NGFI-B was not regulated during reconsolidation. This consolidation-specific expression of NGFI-B shows that reconsolidation is not a mere recapitullation of consolidation. This conclusion is in agreement with earlier indications that reconsolidation might differ from consolidation. For example, a higher dose of anisomycin is required to block contextual fear memory reconsolidation in comparison to consolidation (Debiec et al., 2002). Furthermore, reconsolidation appears to be a faster process than consolidation (Anokhin et al., 2002; Judge and Quartermain, 1982).

NGFI-B up-regulation during memory consolidation was specific to the context-shock association. One may thus argue that the lack of NGFI-B regulation during memory reconsolidation is due to the absence of the shock during the re-exposure, as the context-shock association is no longer present. However, the expression of SGK3 during consolidation was also specific to the context-shock association, and yet, SGK3 was up-regulated during reconsolidation. Thus it would be difficult to interpret the lack of NGFI-B up-regulation be due to the lack of US sttimulus. It is more plausible that NGFI-B is important for establishing the original memory trace but is not required for reconsolidation, whereas SGK-3 is required for both.

Recently, a study using antisense oligodeoxynucleotides showed that BDNF was required for consolidation of contextual fear memory, but not for reconsolidation, whereas zif268 was required for reconsolidation of contextual fear memories, but not for consolidation, thus also demonstrating a dissociation between consolidation and reconsolidation (Lee et al., 2004). However, although the study demonstrated that consolidation and reconsolidation differ, it did not discuss whether consolidation and reconsolidation are mediated by completely different molecules, or whether there are overlapping uses of molecules but, used in different ways by the two processes. Interestingly, the same authors had previously shown that zif268 is engaged during both consolidation and reconsolidation of contextual memory, showing that zif268 is not reconsolidation-specific and suggesting that there are at least some overlapping uses of molecules (Hall et al., 2000; Hall et al., 2001).

Taking into consideration the findings of this study, together with all the data published in the field, the following hypothesis is put forward: at the molecular level, reconsolidation is a partial recapitulation of consolidation, engaging only a subset of processes involved in memory consolidation. Therefore during consolidation, there might be functional redundancies that do not occur during reconsolidation. Consistent with this idea, the up-regulation of functionally redundant transcripts was observed during consolidation in the present study. Inhibiting a gene during consolidation might have no obvious effect on LTM formation, if proteins performing the same role are also expressed. However, inhibiting this same gene during reconsolidation might have more deleterious effects, as fewer genes are engaged in that phase.

4.5 CONCLUSION

This study shows for the first time that both αCaMKII autophosphorylation-dependent and –independent transcriptions occur during contextual fear memory consolidation. Furthermore, it demonstrates that expression changes specific to the context-shock association can be recapitulated during reconsolidation. This is consistent with the idea that the reconsolidation processes triggered by contextual fear memory retrieval are engaged again in subsequent encoding of the reactivated context-shock memory trace. In addition, this study shows that reconsolidation processes in the hippocampus change over time, since retrieval of remote memories does not trigger the transcriptional changes induced by retrieval of recent memories. Finally, this study demonstrates that memory reactivation induces only a subset of the transcriptional events occurring during consolidation. Thus, the following hypothesis is put forward: memory reconsolidation is a partial recapitulation of memory consolidation. Such partial rather than total recapitulation may have evolved as a more economic and reliable mechanism for an organism to modify a memory.

APPENDIX 1:
DIFFERENTIAL DISPLAY PRIMERS

Anchor primers

5'-TTTTTTTTTTTTVA-3'

5'-TTTTTTTTTTTTVC-3'

5'-TTTTTTTTTTTTVG-3'

Where V is a mixture of A, C and G

Arbitrary Reverse (AR) Primers:

AR1	5'-CAGTCAGCAA-3'	AR15	5'-TGCGAGAATG-3'
AR2	5'-TGACGTCGAA-3'	AR16	5'-TTTCCCCTTG-3'
AR3	5'-GGAGAGAACA-3'	AR17	5'-CTGCTAGCAT-3'
AR4	5'-CATGTCCTCA-3'	AR18	5'-TGCTTGCGAT-3'
AR5	5'-TTGCCCAAGA-3'	AR19	5'-GGGGAAAACT-3'
AR6	5'-GGAATGCTGA-3'	AR20	5'-CAAGCCTTCT-3'
AR7	5'-TCCGAAGCTA-3'	AR21	5'-AGTACCGAGT-3'
AR8	5'-GCCCTTTGTA-3'	AR22	5'-GGATGCTTGT-3'
AR9	5'-AGGTCGAAAC-3'	AR23	5'-TCCGGAACTT-3'
AR10	5'-TCACGCTTAC-3'	AR24	5'-TAGTCCCGTT-3'
AR11	5'-CGAGGAAATC-3'	AR25	5'-ACTGACTACC-3'
AR12	5'-GATGCTCTTC-3'	AR26	5'-GTCATGATCC-3'
AR13	5'-TTCCCGAAAG-3'	AR27	5'-TTCTCTCCAC-3'
AR14	5'-CGAAGTCTAG-3'	AR28	5'-ACGTGAAGAC-3'

APPENDIX 2:
PRIMERS USED FOR QPCRS

Primer	Sequence
5HTr4-FWD	5'-GGGCCCCCTTCTTTGTCA-3'
5HTr4-REV	5'-ATAGCCAAGCCAGAGGAAAGC-3'
BDNF-FWD	5'-CCAAAGGCCAACTGAAGCA-3'
BDNF-REV	5'-CGAGTTCCAGTGCCTTTTGTC-3'
CLONE I9-FWD	5'-AGAGGGAAAGATGATGGTTGGA-3'
CLONE I9-REV	5'-GTGCCGTTAGTAAGTTGGTAATGGA-3'
CLONE P7-FWD	5'-AGACACAGATGTACAAGAGCCTCACT-3'
CLONE P7-REV	5'-AAATTCCGGTACTTCCTGCCTTA-3'
CYCLIN C-FWD	5'-GGACATGGGCCAGGAAGAC-3'
CYCLIN C-REV	5'-CGATCATGAACGGAGGGTACA-3'
GAPDH- REV	5'-CAGGAAATGAGCTTGACAAAGTTG-3'
GAPDH-FWD	5'-AGGTTGTCTCCTGCGACTTCA-3'
HPRT-FWD	5'ATACAGGCCAGACTTTGTTGGATT3'
HPRT-REV	5'-TCACTAATGACACAAACGTGATTCAA-3'
MKP1-FWD	5'-ACAACCACAAGGCAGACATCAG-3'
MKP1-REV	5'-CGGCCTGGCAATGAACA-3'
NELL1-FWD	5'-CCATCAGAGGAAGGCGTTTG-3'
NELL1-REV	5'-AACAACCATCGACCGAAATACAC-3'
NGFIB-FWD	5'-TGGCTTTGGTGATTGGATTGA-3'
NGFIB-REV	5'-GGAGCCCGTGTCGATCAGT-3'
NOR1-FWD	5'-CGCCGAAACCGATGTCA-3'
NOR1-REV	5'-TGTACGCACAACTTCCTTAACCA-3'
NOT2-FWD	5'-TGTCGCCCTGTGCACTAAAG-3'
NOT2-REV	5'-GTGAATTGTCTCAGTTCACGTCTTG-3'
NURR1-FWD	5'-GGACCTGCTTTTTGAATCAGCTT-3'
NURR1-REV	5'-CACCCCATTGCAAAAGATGAGT-3'
PREALBUMIN-FWD	5'-TCCATGAATTCGCGGATGT-3'
PREALBUMIN-REV	5'-AGCCGTGGTGCTGTAGGAGTA-3'
SGK1-FWD	5'-TTCTGAACAAGCCTCTCCAGTTG-3'
SGK1-REV	5'-GGCACCCAGCCTCTTGGT-3'
SGK3-FWD	5'-TTCACTGAAGAAACGGTTCCCTAT-3'
SGK3-REV	5'-AAAAACCAACAAATGCATCATCTG-3'
TOB1-FWD	5'-CAACCTCAGTCCCCTCCAGTAC-3'
TOB1-REV	5'-TTAAGCTAAAATTCAAGCCGTCTACA-3'

APPENDIX 3:
FINAL PRIMERS CONCENTRATION FOR qPCRS

Primer	Concentration Fwd/Rev (nM)	Endogenous control	Concentration for control Fwd/Rev (nM)
5HTr4	300/900	GAPDH	300/300
BDNF	300/300	GAPDH	300/300
CYCLIN C	300/900	HPRT	900/900
Clone I9	900/900	HPRT	900/900
MKP1	900/900	HPRT	900/900
NELL1	900/900	GAPDH	300/300
NGFIB	900/300	HPRT	900/900
NOT2	900/300	GAPDH	300/300
NURR1	900/900	HPRT	900/900
Clone P7	900/900	GAPDH	300/300
PREALBUMIN	300/300	GAPDH	300/300
SGK1	900/300	HPRT	900/900
SGK3	300/300	HPRT	900/900
TOB1	300/300	HPRT	300/300
NOR-1	900/300	HPRT	900/900

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